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We recently completed a case-control study nested within a cohort of 4,888 women with benign breast disease (BBD) who participated in the National Breast Screening Study (NBSS). This showed that accumulation of p53 protein was associated with an increased risk of progression to breast cancer (adjusted odds ratio (OR) 2.55; 95% confidence interval (CI) 1.01-6.40), whereas c-erbB-2 protein overexpression was not (adjusted OR 0.65; 95% CI 0.27-1.53). In this grant, we proposed to: (1) collect paraffin-embedded breast tissue the remaining 4,336 (that is 4,888-552) cohort members; (2) enlarge our ongoing case-control study with an additional 63 cases (and their controls) which were identified as a result of a linkage of the NBSS database to the Canadian Cancer Database; (3) examine whether cyclin D1 gene amplification or protein overexpression determined immmunohistochemically is a biomarker of increased risk of breast cancer. To date, we have updated the data base and are collecting blocks for the remaining cohort members. Our initial analysis suggests that cyclin D1 overexpression, as detected immunohistochemically, does not appear to be associated with increased risk of developing breast cancer (OR 1.2; CI 0.65-2.29) in the individuals studied to date. Further analysis is ongoing. This study will be expanded when the additional cases are identified by the linkage described above.

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Rita Kandel 9/10/99

PI - Signature Date

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INTRODUCTION

We have completed a study of the associations between c-erbB-2 protein overexpression and p53 protein accumulation in benign breast tissue and the risk of subsequent breast cancer (1). The study was conducted as a case-control study nested within the cohort of 4888 women in the National Breast Screening Study of Canada (NBSS) who were diagnosed with benign breast disease and underwent active follow-up. Case subjects were the women who subsequently developed breast cancer (ductal carcinoma in situ (DCIS) or invasive carcinoma). Control subjects were matched to each case subject by NBSS study arm, screening center, year of birth, and age at diagnosis of benign breast disease. Histologic sections of benign and cancerous breast tissues were analyzed immunohistochemically. Information on potential confounding factors was obtained by use of a self-administered lifestyle questionnaire completed at the time of enrolment. Accumulation of p53 protein was associated with an increased risk of progression to breast cancer (adjusted odds ratio (OR) = 2.55; 95% confidence interval (CI) = 1.01-6.40), whereas c-erbB-2 protein overexpression was not (adjusted OR = 0.65; 95% CI = 0.27-1.53). The findings for c-erbB-2 and p53 did not differ among stratas defined by menopausal status, allocation within the NBSS, history of breast disease, and whether the benign breast disease was detected at a scheduled screen or between screens. The results were also similar after exclusion of case subjects whose diagnosis of breast cancer occurred within 1 year of their diagnosis of benign breast disease and after exclusion of subjects with DCIS. In summary, p53 protein accumulation, but not c-erbB-2 protein overexpression, appeared to be associated with an increased risk of progression to breast cancer in women with benign breast disease. The current work funded by the U.S. Army Medical Research and Materiel Command will allow us to enlarge this case-control study, study changes in cyclin D1 in the same individuals, and to create a tissue bank for further studies.

The purpose of this project is threefold. Specifically, we are:

(1) collecting paraffin-embedded benign breast tissue from the remaining 4,336 cohort members who are not part of the case-control study. This will establish a bank of paraffin-embedded tissue for a cohort of women on whom there is extensive documentation of risk factor information. With further follow-up of the cohort, it will be possible to enlarge the above described case-control study, and to undertake additional studies of newly identified molecular markers of risk of progression to breast cancer.

(2) enlarging our recently completed case-control study of p53 with an additional 63 cases (and 5 matched controls per case) which were identified as a result of a linkage of the NBSS database to the Canadian Cancer Database. We propose to examine biopsies from these subjects for evidence of c-erbB-2 overexpression and p53 protein accumulation. Addition of these cases and controls to the previous study will increase its statistical power. As well, we will examine the tissue for the presence of p53 mutations in these group of women.

(3) examining whether cyclin D1 amplification and/or protein overexpression determined immmunohistochemically is a molecular marker of risk of progression from BBD to

breast cancer in the enlarged case-control study. We hypothesize that cyclin D1 overexpression in benign breast disease is associated with increased risk of progression to breast cancer.

Epithelial cancers appear to be the result of an accumulation of multiple genetic events (2-4). Multiple molecular markers are being examined, since progression to cancer probably results from the accumulation of several genetic events (5). To date, in relation to breast cancer, cyclin D1 and c-erbB-2 overexpression, and p53 mutations are amongst the more frequent genetic changes detected. While cyclin D1, c-erbB-2, and p53 appear to play an important part in mammary carcinogenesis, their precise role in this process is unclear. For example, it is unclear whether they are involved in the initiation of transformation or at a subsequent stage, or whether they are just indicators of increased risk of developing breast cancer as they may be markers of genetic instability. Further study will be required to determine their contributions.

BODY

(1) STUDY DESIGN:

Our study uses paraffin-embedded breast tissues which have been obtained from the cohort of women enrolled within the National Breast Screening Study (NBSS) who received a diagnosis of benign breast disease during the active follow-up phase of the study. In the ensuing paragraphs, we describe the NBSS first, and then present details of the collection of paraffin blocks and subsequent investigations.

(a) The National Breast Screening Study: The NBSS is a multi-center randomized controlled trial of screening for breast cancer in Canadian women aged 40 to 59 at recruitment (6,7). The study involves 89,835 women who were recruited at 15 screening centers across Canada. Recruitment commenced in 1980 and ended in 1985. Women were eligible to participate in the study if they had no history of breast cancer, were not currently pregnant, and had not had a mammogram in the preceding 12 months.

Women aged 40-49 years were randomized either to have annual mammography plus physical examination, or to have initial physical examination only, and women in both the intervention and the control group were taught breast self-examination. Randomization in the 50-59 year age-group was either to annual mammography plus physical examination, or to annual physical examination alone (women in this arm of the 50-59 year age-group were also taught breast self-examination).

(i) Diagnosis of BBD and breast cancer in the NBSS: At each visit, study participants had a physical examination. For those who were randomized to the intervention group, physical examination was followed by mammography, the films from which were read by a study radiologist who was unaware of the physical examination results. If the examiner or the radiologist reported an abnormality requiring further assessment, a referral was made to a review clinic where the participant was seen by a study surgeon. If, on review, a recommendation was made for biopsy, this recommendation was conveyed to the participant's family physician, and the participant was contacted and asked to visit her family physician for further management.

Women in both control groups were referred for mammography if either they or their primary care physician discovered an abnormality for which referral was warranted. Staff in each screening center identified the pathology laboratory in which biopsies were examined, and they obtained slides or blocks for review by a locally designated reference pathologist. Results of the histological review of the biopsies were forwarded to the coordinating center.

(ii) Follow-up in the NBSS: Active follow-up continued until 1988. During this phase of the NBSS (when the study participants underwent the screening schedule corresponding to their allocation, as described in (a) above), there was in each study center a coordinator (usually a nurse) who had experience in clinic or study management. The coordinators were responsible for ascertaining whether the

recommended diagnostic procedures had been carried out and for collecting reports of the surgical and pathological procedures from the institutions where they had been performed. Procedures performed independently of the screening process were identified through annual questionnaires sent to study subjects, and reports of these procedures were then obtained from the relevant institutions. Study records for women who moved out of their original area were transferred to the center nearest their new residence. Following completion of their screening schedule, direct follow-up stopped for those with no diagnosis of breast cancer. However, until 1988-1990 (depending upon the province) information about new diagnoses of breast cancer was obtained by linkage with the provincial cancer registries (cancer is registered in each province in Canada, and, for Ontario at least, registration is essentially complete (8)). Subsequently, new diagnoses of cancer will be ascertained by linkage to the Canadian Cancer Database, which is operated by the Canadian Center for Health Information at Statistics Canada, and consists of registration data reported annually by the provincial registries. A linkage yielding incidence data to the end of 1993 was completed recently, and we propose that another linkage take place in mid 2000 to yield a further four to five years of follow-up data.

(b) Description of the cohort: The immunohistochemical and molecular investigation currently underway is being undertaken within the cohort of 4,888 women within the NBSS who received a histopathologic diagnosis of BBD during the active follow-up phase of the NBSS. In order to reduce costs substantially while having relatively little impact on the precision of the estimates of association (9), the study is being conducted as a case-control study nested within this cohort. Cases are women who subsequently developed breast cancer, while controls are women who had not developed breast cancer by the date of diagnosis of the corresponding case. Five controls were selected for each case, and they are matched to the corresponding case on study arm within the NBSS, screening center, year of birth, and age at diagnosis of BBD.

(i) Case definition: Cases are women with a history of BBD detected during the course of the NBSS who subsequently developed breast cancer. By this definition, 92 cases were identified by previous linkages. We collected the benign tissue from 74 cases. As described below, as a result of the linkage to the 1993 database, we have identified 63 additional cases.

(ii) Definition of controls: Controls are women who had not developed breast cancer by (but were alive at) the date of diagnosis of the corresponding case (they will, of course, have a diagnosis of BBD). Since there are no estimates of the likely magnitude of the effects of interest on risk of progression from BBD to breast cancer, we select 5 controls for each case in order to maximize statistical power. Controls are matched to cases on study arm within the NBSS, screening center, year of birth, and age at diagnosis of BBD (and implicitly on the interval between diagnosis of BBD and the date of diagnosis of breast cancer in the corresponding case). These matching criteria are chosen either because the factors of interest are related to breast cancer risk (age, and possibly age at diagnosis of BBD) or because they are related to the risk of disease detection (allocation and screening centre). It is also conceivable that at least some of these factors are related to the exposures of interest. However, it should be noted that

little is known about the "epidemiological" correlates of cyclin D1 and c-erbB-2 overexpression, and p53 protein accumulation. Additionally, the implicit matching on duration of follow-up (as well as age) means that the controls have had the same opportunity (at least, in terms of the elapse of time) to develop breast cancer as the cases.

(iii) Questionnaires: At the time of their enrolment in the NBSS, all participants completed a questionnaire which sought identifying information, as well as data on factors such as demographic characteristics, family history of breast cancer, menstrual and reproductive history, use of oral contraceptives and replacement estrogens, and cigarette smoking. Additionally, approximately two-thirds of the 89,835 women enrolled in the NBSS completed self-administered diet history questionnaires. The dietary questionnaire was introduced in 1982, at which time some women had already been enrolled in the study (and were not seen again at the screening centers). The diet history contained questions on the frequency of consumption and usual portion size of 86 food items, and also had an open-ended section for describing other food items normally eaten. Photographs of various portion sizes were included in the questionnaire to assist participants to quantify intake. The questionnaire also included questions on current and past height and weight, and on consumption of beer, wine, and spirits. A comparison between the self-administered questionnaire and a full interviewer-administered questionnaire which has been subjected to both validity and reliability testing (10) and used in a number of epidemiologic studies (11) revealed that the two methods give similar results for the major macronutrients, dietary fiber, and vitamin C (12).

(iv) Statistical power: This was calculated according to that described by Breslow and Day (13).

(2) CONDUCT OF THE STUDY:

(a) Coding, data entry, and processing: The lifestyle information is available on the computerized NBSS database. The standard procedures of the Cancer Epidemiology Unit for quality control are used for coding and data entry.

(b) Collection of paraffin-embedded breast material: For the completed case-control study of p53 and c-erbB-2 protein changes in benign breast disease, we created a database consisting of identifying information, plus details of the location and accession number of the 552 paraffin blocks. This information was used to generate lists for each hospital of the study participants for whom we wished to obtain paraffin blocks. We then wrote to the pathologist-in-chief at the hospital seeking the blocks.

This same approach was used to expand the existing tissue bank. The database was updated to include all 4,888 subjects with a diagnosis of BBD in the NBSS. We are currently attempting to collect the blocks of the remaining 4,336 (4,888-552) women.

(c) Histopathological Review: Sections from blocks received for the expanded nested case-control study will be reviewed and classified by Dr. Kandel and a collaborator, Dr.

W. Hartwick, according to the criteria developed by Page (14), and as described in the consensus conference for DCIS (15). Briefly, in benign lesions, the presence or absence of epithelial proliferation is determined, and when epithelial proliferation is present, the lesions were classified further according to the presence or absence of cytological atypia. The cancers are classified by histological type.

(d) Experimental methods: In this section we describe the methodology that was used to evaluate cyclin D1 gene amplification and protein overexpression in the nested cohort of women that was used to assess p53 and c-erbB-2. For completeness we also present details of the immunohistochemical staining for c-erbB-2 and p53 as well as the molecular analysis of p53 which will be done on the newly identified subjects who show p53 protein accumulation.

(i) Cyclin D1 in Breast Tissue:

Cyclin D1 immunohistochemistry

Since we do not have access to frozen tissue, immunohistochemical staining was used to detect cyclin D1 overexpression. The antibody that we selected works on paraffin-embedded tissue. There is a good correlation between immunostaining and Western blot analysis which indicates that the positive immunoreactivity is not a false positive (16). Immunohistochemical staining allows cellular localization of the immunoreactivity, so it was possible to ensure that the cyclin expression was occurring in breast epithelial cells. In addition, this approach allowed us to determine whether the immunoreactivity was present in the histopathology considered to be associated with increased malignant potential. Breast cancers were stained in order to determine whether the expression present in the benign breast disease was maintained in the malignant lesion, or was present in the breast cancer only.

Tissue sections were placed on 2% aminopropyltriethoxysilane (Sigma)-coated slides and deparaffinized. The tissue underwent antigen retrieval (microwave pretreatment in 10 mM citrate buffer, pH 6.0, for 15 minutes at a medium-high setting). The endogenous peroxidase was inactivated using 3% hydrogen peroxide, and the sections were blocked with normal horse serum (20 μL/mL Vector Laboratories, Burlingame, CA) containing 5% bovine serum albumin (BDH Laboratory, Poole, England) in Tris-buffered saline (5 mM Tris-HCl (pH 7.6) and 0.85% sodium chloride). The sections were incubated overnight at 4°C with antibody reactive with cyclin D1 protein (monoclonal, dilution 1:2000; Upstate Biotechnology, Lake Placid, NY). After washing, the sections were incubated with biotinylated antimouse immunoglobulin G (dilution 1:200; Vector Laboratories) for 30 minutes at room temperature, followed by avidin-biotin peroxidase complex (Vectastain Elite ABC Kit; Vector Laboratories). Immunoreactivity was visualized with 3,3'-diaminobenzidine (Vector), and the sections were counterstained briefly with hematoxylin. T47D cells embedded in paraffin served as the positive control (17). The negative control consisted of replacing the primary antibody with Tris-buffered saline or nonimmune mouse serum (DAKO, Carpinteria, CA). Distinct nuclear staining in greater than 1% of epithelial cells indicated a positive reaction and cytoplasmic staining was considered nonspecific and interpreted as negative.

Cyclin D1 amplification

Five um thick sections were cut, briefly stained with hematoxylin. The epithelium in the tissue which showed cyclin D1 immunoreactivity was microdissected out and placed in a microfuge tube. The tissue sections showing no cyclin D1 protein accumulation immunohistochemically underwent random microdissection of epithelium. DNA was extracted by incubating the microdissected tissue in buffer (50 mM Tris-HCl, pH 8.5, 1 mM ethylenediamine tetracetic acid, 0.5% Tween 20) containing 0.5 mg/ml of proteinase K (Sigma Chemical Co, St. Louis, MO) at 50°C for 48 hours. The proteinase K was then inactivated by boiling at 95°C for 15 minutes.

Semiquantitative differential polymerase chain reaction (PCR) was used to determine the presence of cyclin D1 gene amplification and to estimate its extent. As fragmented genomic DNA (<200 bp) may influence the results of differential PCR, γ-interferon (γ-IFN) was analyzed in a multiplex PCR reaction in order to indirectly assess DNA quality first (18,19). Two sets of primers, specific for different exons of γ-IFN gene and which generate PCR products of 150 and 82 bp (γ-IFN 150 and γ-IFN 82) were co-amplified in the same reaction tube as described previously (19). If the γ-IFN82/γ-IFN150 ratio of the PCR products was 3 or less, the tissue was considered suitable for further analysis. For such cases, aliquots of the proteinase K digested tissue were then examined for cyclin D1 amplification using PCR. Both dopamine receptor (DR) and cyclin D1 were co-amplified in the same reaction tube. DR was chosen as it is present on the same chromosome cyclin D1 (20). If the tissue had chromosomal duplication it would simulate amplification and would be a false positive. To prevent this we selected a gene on the same chromosome yet of sufficient distance from cyclin D1 that it was unlikely to be part of an amplified amplicon. Included in each run was DNA extracted from two paraffin embedded cell lines; MDA-MB-231 which shows no gene amplification and ZR-75-1 which has cyclin D1 amplification (21,22). Briefly, 1 µl of the digest was mixed with 14 µl of PCR working solution containing 10 mM Tris Hcl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 100 µM of each dNTP, 1 U of AmpliTaq DNA polymerase (Roche Diagnostic Systems Inc., Branchburg, NJ) and 1 µM of each primer. The samples underwent 30 cycles of amplification in an automated thermocycler (DNA Thermal Cycler, Perkin Elmer, Branchburg, NJ). Each cycle consisted of 1.2 minutes of denaturation at 94°C, 1 minute of annealing at 55°C, and 1 minute of elongation at 72°C. The PCR products were separated by electrophoresis on a 12% polyacrylamide gel at 200V for 2 hours and visualized following ethidium bromide staining. Each tissue was analyzed at least twice in separate polymerase chain reactions. Each sample showing amplification of cyclin D1 was then repeated one additional time. Concurrently, DNA extracted from the subject's breast stomal tissue, which should show no amplification, was also analyzed as a control. Direct sequencing of selected PCR products using the sense primer and the Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham Life Sciences, Cleveland, Ohio, USA) confirmed the specificity of the primers (23).

To determine whether there was cyclin D1 gene amplification, the ratio of the cyclin D1 PCR product to the DR PCR product was derived from photographic

negatives of ethidium bromide stained gels which were quantified by laser densitometry (Computing Densitometer Model 300A, Molecular Dynamics, Sunnyvale, CA). There were at least two gels per sample and each gel was scanned three times. A mean ratio of cyclin D1 to DR was determined and a ratio of greater than 0.88 was considered indicative of gene amplification. This value was determined by calculating two standard deviations from the average of the ratios (n=13) obtained from the negative control cell line that had no gene amplification.

(ii) Assessment of p53 accumulation and c-erbB-2 overexpression in breast tissue

The subjects newly identified by the recently completed linkage will be used to expand the previously published study (1). The paraffin blocks from these individuals, if retrievable from the hospital, either are already in our possession, or will be shortly. Tissue sections from these blocks will undergo immunostaining for p53 and c-erbB-2 using standard immunohistochemical methods as described previously (1).

(iii) Methodology to detect p53 mutations in breast tissue

As well we will determine whether p53 mutations occur in the tissues from the subjects newly identified by the recently completed linkage that will be used to expand the previously published study (1). The DNA will be extracted from the tissue that shows p53 protein accumulation immunohistochemically in the newly identified 63 cases and their corresponding controls and the DNA will be examined for the presence of mutations in exons 4 to 9.

Five µm sections will be cut from the paraffin blocks dewaxed and stained briefly with hematoxylin. The epithelium in the region of the tissue that shows p53 immunoreactivity will be microdissected out and placed in a microfuge tube. The tissue will be digested with proteinase K (0.5 mg/ml in 50 mM Tris HCl, pH 8.5, 10 mM EDTA, 0.5% Tween 20) for at least 48 hrs at 55°C (23). The proteinase K will be inactivated by heating at 95°C for 15 min.

An aliquot of the digest will be amplified using PCR, [α-^{33}P]-dATP and exon-specific primers. An aliquot of the reaction product will be separated on an 8% non-denaturing polyacrylamide gel and the gel will be processed for autoradiography (24,25). Potential mutations are detected by shifts in band mobility. If no band shifts are detected in these samples, the tissue will be considered to have no mutation. For samples showing band shifts, the PCR-SSCP analysis will be repeated. If the two PCR-SSCP analyses generate different band shifts, another section will be cut, microdissected and processed for PCR-SSCP analysis as described above. Negative controls including cells which contain no mutation and a blank water control are included in each analysis. In addition positive controls for exons 5 to 9 (exon 5:SKBr 3; exon 6:T47D; exon 7:colo 320 DM; exon 8: MDAMB468; exon 9: SW480) were also included where appropriate. The cell lines used as positive controls will be embedded in agar, fixed in 10% formalin, and paraffin-embedded to simulate the processing conditions of the breast tissue.

The abnormally shifted band will be excised from SSCP gels and the DNA eluted into water. The DNA will be reamplified by PCR using the same primers and the product run on a 2% agarose gel. The band will be extracted using QIAquick gel extraction kit (Qiagen Inc, Mississauga, ON). The purified DNA will be manually sequenced using ThermoSequenase radiolabelled terminator cycle sequencing kit (Amersham Life Sciences, Cleveland, OH) and the sense primer according to the manufacturer's directions, followed by gel electrophoresis and autoradiography. To confirm the mutation, the DNA product will be resequenced using the antisense primer. Negative controls will be included in each analysis. Cell lines with known mutations in exons 5 to 9 are also included where appropriate. Gene alterations will be compared to those listed for breast cancer in a p53 database (http://www.iarc.fr/p53).

(e) Statistical analysis: Essentially, the statistical analysis involves comparison of the frequency (either singly or in combination) of cyclin D1, c-erbB-2 overexpression and p53 protein accumulation and mutations in the cases and controls, using conditional logistic regression with multiple controls per case (9). The association between these genetic changes and factors which are thought to be involved in the etiology of BBD and breast cancer (e.g., reproductive, menstrual, and dietary factors, as well as BBD histology) are examined, as well the association of the latter variables with risk of progression to breast cancer.

Further analyses will be directed towards within-individual comparisons of cyclin D1 and c-erbB-2 overexpression and p53 in BBD and breast cancer. One possible interpretation of any changes which are found to be common to both conditions will be that they contribute to the development of breast cancer rather than arise as a consequence of it.

RESULTS

We have updated the database with respect to identifying details of the remaining individuals in the cohort with benign breast disease.

We have contacted a total of 253 hospitals. The number has changed from our grant proposal because of the ongoing hospital mergers that are occurring in Canada. We are in the process of accessioning the paraffin blocks received to date. 151 hospitals/ laboratories have sent 2015 blocks out of 2635 requested (76%). We are in contact with 23 hospitals of which 9 have agreed to send blocks (269 blocks) but have not done so yet. The remaining 14 hospitals are in discussion with us and have indicated that they may be willing to send the blocks that we have requested (412 blocks). Fifty-four hospitals (1153 blocks) have replied and informed us that the blocks requested have been discarded and 25 hospitals had sent the blocks to other locations. Repeated follow-up phone calls are being made to the lab director or their designate for the outstanding 23 hospitals.

Cyclin D1 immunostaining of tissue sections from the 357 existing blocks of benign breast disease has been completed. Cyclin D1 overexpression was seen in 76 samples of benign breast disease. Seventeen of the immunopositive tissues were cases and 59 were controls. When the analysis was completed the presence of cyclin D1 overexpression was not associated with increased risk of developing breast cancer (unadjusted odds ratio = 1.2, 95% confidence intervals = 0.65-2.29). Twenty-two of 52 cancers showed cyclin D1 overexpression. Ten of the 22 immunopositive cancers had cyclin D1 overexpression in their corresponding benign tissue. Six cancers that were immunonegative showed cyclin D1 overexpression in their benign tissue. The immunostained sections are being re-reviewed and these results finalized. Data analysis is continuing.

Cyclin D1 amplification was assessed in DNA obtained from breast tissue sections from 358 subjects. In one subject, no DNA could be obtained from the tissue and for 23 subjects there was insufficient breast epithelium present to warrant microdissection. Gene amplification was detected in 41 subjects and amplification values ranged from 0.89 to 1.27. Previous studies have shown that the breast cell line ZR-75-1, which was used as positive control, had approximately a three-fold amplification (26,27) and this cell line using our methodology had on average an amplification ratio of 1.26 ± 0.96. This suggests that when cyclin D1 in the breast tissues was amplified it had at most three-fold amplification.

In the third year of this grant, tasks 1 and 2 of technical objective 1 are either completed (task 1) or almost completed (task 2) and are within the timelines indicated in the revised statement of work. Tasks 3 and 4 of technical objective 2 are completed. Task 5 is almost complete and should be completed as indicated in the revised statement of work.

However, technical objective 3 (tasks 6 to 9) in the statement of work in the grant proposal has not been accomplished yet because of circumstances beyond our control. This task involves extension of the ongoing project by the addition of more cases and their controls identified as a result of longer follow-up. The cases are identified by the linkage of the NBSS database to the Canadian Cancer Database and this was delayed at Statistics Canada. Once the linkage was done the breast cancer diagnoses had to be verified and this took more time than anticipated for technical reasons. However, all this has been completed and an additional 63 cases were identified as a result of that linkage and will result in a total of 155 cases if we are able to obtain all of the blocks. We are currently identifying the controls for these cases. We anticipate starting the work described in technical object 3, namely immunostaining for p53, c-erbB-2, and cyclin D1, within 2 months.

In anticipation of starting the experimental work for the expanded study, we have developed the methods to detect p53 mutations in DNA extracted from paraffin embedded tissue (task 7). This work is the subject of a manuscript that we have submitted to International Journal of Cancer. We are also now attempting to determine whether automated sequencing can be utilized on DNA extracted from paraffin-

embedded tissue. The methodologies required to do tasks 6 and 8 are already established.

Our last technical objective (#4) entails preparing a file for a second linkage to obtain patient clinical follow-up to the year 1997-98. The file is being prepared and should be transferred to Statistics Canada by May 2000 which is within the time frame stated in the revised Statement of Work.

KEY RESEARCH ACCOMPLISHMENTS:

- creation of a tissue bank of benign breast tissue
- demonstrated that cyclin D1 protein overexpression is not associated with increased breast cancer risk
- demonstrated that cyclin D1 gene amplification occurs in normal and benign breast tissue
- determining whether cyclin D1 gene amplification is a marker of increased breast cancer risk
- demonstrated that p53 mutations and gene changes occur in normal and benign breast tissue
- expansion of cohort study which should refine the role of p53 protein accumulation as a marker of increased breast cancer risk

REPORTABLE OUTCOMES:

A) The following publications and abstracts have resulted from the work supported by this grant.

1. Zhu XL, Rohan T, Hartwick W, Kandel R. Cyclin D1 gene amplification and protein expression in benign breast disease and breast carcinoma. Mod Pathol 11: 1082-1088, 1998.

2. Duffy SW, Rohan TE, Kandel R. Misclassification in a matched case-control study with variable matching ratio. Submitted to Statistics in Medicine, 1999.

3. Kandel R, Li, S-Q, Ozcelik H, Rohan T. p53 protein accumulation and mutations in normal and benign breast tissue. Submitted to International Journal of Cancer, 1999.

4. Rohan T, Zhu X-L, Kandel R. Cyclin D1 in benign breast disease and risk of breast cancer. Proceedings of the American Association for Cancer Research, Philadelphia, PA, April 10-14, 1999.

5. Pollett AF, Bedard YC, Rohan T, Kandel RA. Detection of p53 mutations in ThinPrep® processed fine needle aspirates of breast carcinoma. American Association of Cytopathology, Acta Cytol 43: 922, 1999.

B) In addition we applied for and received another grant from the US Army Medical Research and Materiel Command (Are p53 mutations associated with increased risk of developing breast cancer? A molecular epidemiological study. #BC980784) that will allow the continuation of this project. This grant focusses on determinining whether p53 gene changes are associated with increased risk of developing breast cancer in the entire cohort.

C) With the support of this grant we have been able to establish a tissue bank of paraffin-embedded normal or benign breast tissue.

CONCLUSIONS

As the collection of blocks and the expansion of our case-control study have not been completed, the conclusions that can be drawn are limited. Our recently published case-control study involving the use of benign breast tissue from individuals enrolled in the NBBS has been called a "paradigm for future studies of additional biomarkers that may identify women with high risk benign breast disease" in a recent editorial about our studies (28). This supports the approach that we are using to identify biomarkers of increased breast cancer risk and we are continuing to collect the paraffin blocks of the benign breast tissue to be able to do these types of studies.

We and others have shown that cyclin D1 protein overexpression occurs in normal and benign breast tissue (22, 28-30). In the currently study, we found that cyclin D1 protein overexpression as detected immunohistochemically does not appear to be associated with increased risk of developing breast cancer in the group of individuals studied to date. This study will be expanded when additional cases are identified by the linkage described above.

We analyzed the tissue for cyclin D1 amplification by semiquantitative differential PCR. We are currently analyzing the data to determine whether the presence of cyclin D1 gene amplification correlates with increased risk to develop breast cancer.

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APPENDIX

Cyclin D1 Gene Amplification and Protein Expression in Benign Breast Disease and Breast Carcinoma

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Cyclin D1 plays a critical role in regulating cell-cycle progression. Gene amplification and protein overexpression of cyclin D1 have been detected in breast cancer but little is known concerning whether these changes occur in normal breast tissue and in breast lesions associated with increased risk of development of invasive breast cancer. We looked for cyclin D1 gene amplification and protein overexpression in 30 cases of benign breast disease (16 epithelial hyperplasias without atypia and 14 atypical ductal hyperplasias) and 18 ductal carcinomas *in situ* by use of differential PCR and immunohistochemical staining. We compared the resulting frequencies to those in 15 cases of normal breast tissue and 17 invasive ductal carcinomas. We found *cyclin D1* gene amplification in 15% of those with normal breast tissue, 19% of those with epithelial hyperplasia without atypia, 27% of those with atypical ductal hyperplasia, 35% of those with ductal carcinoma *in situ*, and 25% of those with invasive ductal carcinoma; corresponding figures for protein overexpression were 13, 13, 57, 50, and 64%. These results suggest that *cyclin D1* amplification and protein overexpression can occur before histologic alterations are seen but that the frequencies of these changes are higher in histologic lesions with cellular atypia (atypical hyperplasia and ductal carcinoma *in situ*), reaching frequencies similar to those observed in invasive carcinoma.

KEY WORDS: Benign breast disease, *Cyclin D1*, Differential polymerase chain reaction, Immunohistochemistry.

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Cancer has been defined as a proliferative disorder characterized by unregulated cell growth (1). Under normal conditions, progression through the cell cycle is orderly and is regulated by cyclins and their associated cyclin-dependent kinases (cdk) (2, 3). Two major checkpoints exist, one at the G_1-S interface and a second at the G_2-M interface. The former prevents replication of damaged DNA, and the latter prevents segregation of structurally altered chromosomes (4). Disruption at either of these points might play a role in the pathogenesis of malignancy (1). Cyclin D is involved in regulating cell cycle progression from G_1 into the S phase (1). There are three types of cyclin D (D1, D2, and D3), each with its own pattern of tissue-specific expression. These cyclins can form complexes with cdk4 or cdk6, which then phosphorylate the retinoblastoma protein and allow the cells to pass into the S phase. Cells that overexpress cyclin D1 show reduced exit from G_1 to G_0 (quiescent phase), suggesting a role for cyclin D1 at this regulatory point (5). Several observations led to the suggestion that *cyclin D1* amplification and/or overexpression are tumorigenic. First, cell transformation results when *cyclin D1* is transfected with the adenovirus *E1A* oncogene into BRK cells (6). Second, rat fibroblasts transfected with *cyclin D1* have a shortened G_1 phase and form tumors when injected into nude mice (7). Third, mammary hyperplasia and breast cancer develop in transgenic mice that overexpress *cyclin D1* (8). Some researchers suggested that *cyclin D1* is not a dominant oncogene but one that requires the presence of other oncogenes to induce tumors (9), whereas other investigators suggested that *cyclin D1* overexpression enhances gene amplification and might contribute to genomic instability (10).

Cyclin D1 has been studied extensively in breast cancer. *Cyclin D1* amplification has been observed in as many as 33% of breast cancer cell lines and between 11 and 23% of human breast cancers (11–16). Cyclin D1 accumulation, detected immunohistochemically, occurs in as many as 81% of breast cancers, although the frequency seems to be de-

pendent on the antibody used (11, 12, 16). There are relatively few reports, however, of cyclin D1 in normal breast tissue and in breast lesions associated with increased risk of developing invasive breast cancer. Immunohistochemical studies showed that normal human breast epithelium and breast tissue adjacent to breast cancers demonstrate, at most, occasional cells that express cyclin D1 protein (14, 17, 18). An *in situ* hybridization study demonstrated that 18% of benign breast lesions showed cyclin D1 mRNA overexpression (19). *Cyclin D1* gene amplification and overexpression, as well as protein accumulation, also occur in ductal carcinoma *in situ* (DCIS) (20). The human and experimental data suggest that *cyclin D1* amplification and/or protein overexpression might have a role not only in breast cancer but also in the putative early stages of breast neoplasia, such as epithelial hyperplasia, a histopathologic change known to be associated with increased risk of progression to breast cancer (21–26). In this study, we explored the occurrence of cyclin D1 protein expression and gene amplification in a series of normal breast tissue samples, cases with benign breast disease, DCIS, and invasive ductal carcinoma.

MATERIALS AND METHODS

Tissues and Cell Lines

We searched the files of the Department of Pathology, Mount Sinai Hospital, Toronto, Ontario, Canada, for the period from 1990 to 1997 and identified 15 representative cases of normal breast tissue, 30 of benign breast disease (16 epithelial hyperplasias without atypia, 14 atypical ductal hyperplasias), 18 DCISs, and 17 breast carcinomas. The benign cases, as well as the DCISs, were from breast biopsy specimens that did not contain invasive carcinoma. The breast tissue had been fixed in 10% neutral buffered formalin and embedded in paraffin. The hematoxylin- and eosin-stained sections were reviewed by two of the authors (RK, WH) and classified according to the criteria described by Page *et al.* (27) and the 1997 consensus conference on the classification of DCIS (28). The following human breast carcinoma–derived cell lines:

- ZR-75–1 and MDA-MB-453, which have twofold to fivefold amplification of *cyclin D1* (14);
- MCF-7, MDA-MB-468, and MDA-MB-231, which have no *cyclin D1* amplification (13, 14); and
- T47D, which shows cyclin D1 overexpression immunohistochemically (14)

were obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in culture, harvested using trypsin-EDTA (Sigma, St. Louis, MO), and centrifuged to form pellets. The cell pellets were placed in 3% bacto-agar (Difco, Detroit, MI), fixed in 10% buffered formalin, and then embedded in paraffin. Sections 5 μm thick were cut and used as controls for polymerase chain reaction (PCR) and/or immunostaining.

Microdissection

Sections were cut from the paraffin blocks using standard precautions to avoid cross-contamination of tissue between cases. This included cutting one case at a time, changing microtome blades between cases, floating the section in its own water bath, and cleaning the work areas of the microtome with xylene between blocks. The sections were dried at 37° C overnight and then deparaffinized. The sections were stained with hematoxylin for 30 seconds, and the pathologic area was dissected with use of a dissecting light microscope (Laborlux 6000; Leica, Toronto, Canada). For the normal breast tissue sections, random ducts and/or lobules were microdissected. Mineral oil (4 μL) (Sigma) was placed on the microdissected tissue, which was then transferred to a microfuge tube in a pipette tip.

DNA Extraction

Genomic DNA was extracted as described by Zhuang *et al.* (29), with some modifications. Briefly, the microdissected tissue was incubated in 50 μL buffer (50 mM Tris–hydrochloric acid (HCl) (pH 8.5), 1 mM EDTA, 0.5% Tween 20) containing 0.5 mg/mL of proteinase K (Sigma) at 50° C for 48 hours. The proteinase K was then inactivated by boiling at 95° C for 15 minutes.

Cyclin D1 Amplification

Semiquantitative differential PCR was used to assess the presence of *cyclin D1* gene amplification and to estimate its extent. Fragmented genomic DNA (< 200 bp) can influence the results of differential PCR, so interferon-γ (IFN-γ) was analyzed in a multiplex PCR reaction to obtain an indirect assessment of DNA quality first (30, 31). Two sets of primers (Table 1), specific for different exons of the IFN-γ gene and which generate PCR products of 150 and 82 bp (IFN-γ 150, IFN-γ 82) were coamplified in the same reaction tube, as described previously (31). If the ratio of IFN-γ 82 to IFN-γ 150 in the PCR products was 3 or less, the tissue was considered suitable for additional analysis (31). For such cases, aliquots of the proteinase K–digested tissue were then examined by PCR for *cyclin D1* amplification. Both *asparagine synthetase* (*Asp*) and *cyclin D1* (Table 1) were coamplified in the same reaction tube. *Asp* is a housekeeping gene and served as an internal control. PCR was performed in

TABLE 1. Sequences of Polymerase Chain Reaction Primers

Gene	Sequences	Sequence region	Reference
IFN-γ 82	Sense, 5′-GCAGAGCCAAATTGTCTCCT-3′	(nt 2012–2031)	31
IFN-γ 82	Antisense, 5′-GGTCTCCACACTCTTTTGGA-3′	(nt 2074–2093)	31
IFN-γ 150	Sense, 5′-TCTTTTCTTTCCCGATAGGT-3′	(nt 4582–4601)	31
IFN-γ 150	Antisense, 5′-CTGGGATGCTCTTCGACCTC-3′	(nt 4712–4731)	31
Cyclin D1	Sense, 5′-ATGTGAAGTTCATTTCCAAT-3′	(nt 722–741)	32
Cyclin D1	Antisense, 5′-TGGGTCACACTTGATCACTC-3′	(nt 851–870)	32
Asparagine synthetase	Sense, 5′-ACATTGAAGCACTCCGCGAC-3′	(nt 496–515)	44
Asparagine synthetase	Antisense, 5′-CCACATTGTCATAGAGGGCG-3′	(nt 639–658)	44

a total volume of 15 μL. Briefly, 1 μL of the digest was mixed with 14 μL of PCR working solution containing 10 mM Tris-HCl (pH 8.3), 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.01% gelatin, 100 μM of each dNTP, 1 U of AmpliTaq DNA polymerase (Roche Diagnostic Systems, Branchburg, NJ), and 1 μM of each primer. The samples underwent 30 cycles of amplification in an automated thermocycler (DNA Thermal Cycler; Perkin Elmer, Branchburg, NJ). Each cycle consisted of 1.2 minutes of denaturation at 94° C (except for the first cycle, which was 10 minutes in length), 1 minute of annealing at 55° C, and 1 minute of elongation at 72° C. The PCR products were separated by electrophoresis on a 12% polyacrylamide gel at 200 V for 2 hours and visualized after ethidium bromide staining. Each tissue was analyzed at least twice in separate PCRs. Direct sequencing of PCR products of one control and case were performed with the initial sense primers and the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Life Sciences, Cleveland, OH), according to the manufacturer's directions.

Semiquantification of *Cyclin D1* Amplification

To assess whether *cyclin D1* gene amplification occurred, the ratio of the cyclin D1 PCR product to the *Asp* PCR product was derived from photographic negatives of ethidium bromide–stained gels that were quantified by laser densitometry (Computing Densitometer Model 300A; Molecular Dynamics, Sunnyvale, CA). There were at least two gels per sample, and each gel was scanned three times. A mean ratio of *cyclin D1* to *Asp* was calculated, and a ratio of greater than 1.5 was considered indicative of gene amplification (11, 32). The amount of *cyclin D1* gene amplification was categorized as + for a ratio of 1.5 through 2.5, ++ for a ratio between 2.5 and 3.5, and +++ for a ratio of more than 3.5.

Cyclin D1 Immunostaining

Tissue sections were placed on 2% aminopropyltriethoxysilane (Sigma)-coated slides and deparaffinized. The tissue underwent antigen retrieval (microwave pretreatment in 10 mM citrate buffer, pH 6.0, for 15 minutes at a medium-high setting). The endogenous peroxidase was inactivated using 3% hydrogen peroxide, and the sections were blocked with normal horse serum (20 μL/mL) Vector Laboratories, Burlingame, CA) containing 5% bovine serum albumin (BDH Laboratory, Poole, England) in Tris-buffered saline (5 mM Tris-HCl (pH 7.6) and 0.85% sodium chloride). The sections were incubated overnight at 4° C with antibody reactive with cyclin D1 protein (monoclonal, dilution 1:2000; Upstate Biotechnology, Lake Placid, NY). After washing, the sections were incubated with biotinylated antimouse immunoglobulin G (dilution 1:200; Vector) for 30 minutes at room temperature, followed by avidin-biotin peroxidase complex (Vectastain Elite ABC Kit; Vector). Immunoreactivity was visualized with 3,3′-diaminobenzidine (Vector), and the sections were counterstained briefly with hematoxylin. T47D cells embedded in paraffin served as the positive control (14). The negative control consisted of replacing the primary antibody with Tris-buffered saline or nonimmune mouse serum (DAKO, Carpinteria, CA). Distinct nuclear staining indicated a positive reaction and cytoplasmic staining was considered nonspecific and interpreted as negative. In normal tissue, the presence of staining in any of the epithelium was considered positive. In benign breast disease or cancer, only immunoreactivity in the pathologic area was interpreted as positive. The staining was assessed as + when only occasional cells were positive, ++ when clusters of cells were positive, and +++ when there was diffuse staining throughout the pathologic area.

RESULTS

Semiquantification of *Cyclin D1* Amplification

We used the cell lines, ZR-75–1, MDA-MB-453, MCF-7, MDA-MB-468, and MDA-MB-231, to assess the differential PCR assay for sensitivity and reproducibility. The *cyclin D1*-to-*Asp* ratio in the ZR-75–1 and MDA-MB453 cell lines, which according to Southern blot analysis have a twofold to fivefold amplification of *cyclin D1*, was always greater than 1.5. A ratio as high as 5.4 was obtained in some PCR runs. The other cell lines, which are not amplified for *cyclin D1*, had *cyclin D1*-to-*Asp* ratios of less than 1.5 in all runs. These results suggest that this

method is appropriate for determining whether the *cyclin D1* gene is amplified and sufficiently sensitive to detect twofold gene amplification. As shown in Figure 1, a 149-bp product consistent with cyclin D1 was detected in 13 normal samples, 16 hyperplasias, 11 atypical hyperplasias, 17 DCISs, and 16 carcinomas. PCR product sequencing was done for one control (ZR-75–1) and one case (Case C8), which confirmed that the product obtained was cyclin D1. Two normal samples (Cases N14, N15), three atypical hyperplasias (Cases A12, A13, A14), one DCIS (Case D18), and one invasive carcinoma (Case C17) exhibited poor DNA quality: no PCR products for either IFN-γ 82 or IFN-γ 150 were detected (results not shown). As detailed in Tables 2 and 3, amplification was detected in 2 (15%) of 13 cases of normal breast tissue, but, in both of these cases, the level of amplification was low. Three (19%) of 16 epithelial hyperplasias without atypia, 3 (27%) of 11 of atypical ductal hyperplasias, 6 (35%) of 17 DCISs, and 4 (25%) of 16 cancers showed gene amplification. The relative amount of amplification showed no correlation with the histologic changes, because only low levels of gene amplification were detected in breast cancer. Of the DCISs, one of four low-grade tumors, three of six intermediate-grade tumors, and two of seven high-grade tumors showed amplification.

Cyclin D1 Protein Overexpression

Protein overexpression was evaluated by immunohistochemical staining (Fig. 2). We saw cyclin D1 immunoreactivity in two cases (13%) of normal breast tissue (Tables 2 and 3) and two (13%) epithelial hyperplasias without atypia. The proportion of cases showing protein accumulation was higher in the atypical hyperplasias (57%), in the DCISs (50%), and in the invasive cancers (64%) than in the cases of normal breast tissue and epithelial hyperplasia without atypia. In the normal breast tissue, only occasional cells were positive. The intensity and extent of immunostaining was more often greater in the cancers. Nonspecific cytoplasmic staining of epithelial cells, nerves, and/or blood vessels was seen in some sections, but this was easily distinguished from the nuclear staining indicative of protein overexpression.

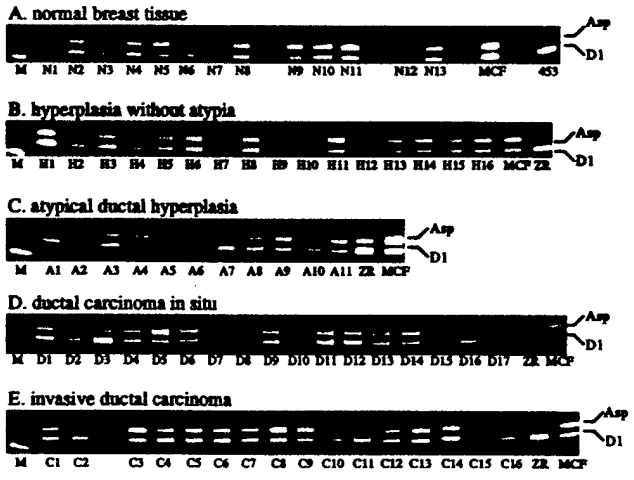

FIGURE 1. Ethidium bromide–stained gel showing PCR products from normal breast tissue (***Cases N1-N13***), epithelial hyperplasia without atypia (***Cases H1-H16***), atypical ductal hyperplasia (***Cases A1-A11***), DCIS (***Cases D1-D17***), invasive ductal carcinoma (***Cases C1-C16***), and controls. The PCR products for cyclin D1 (***D1***) and asparagine synthetase (***Asp***) are indicated. ***M***, 123-bp DNA ladder. ***ZR***, ***MCF***, and ***453*** represent the human breast carcinoma–derived cell lines, ZR-75–1, MCF-7, and MDA-MB-453, which were the controls.

Association of Gene Amplification and Protein Overexpression

Gene amplification occurred in the absence of protein overexpression and *vice versa* (Table 3). One of the 13 normal breast tissue samples had both gene amplification and protein overexpression, whereas 1 of the 16 epithelial hyperplasias without atypia, 2 of the 11 atypical ductal hyperplasias, 4 of the 17 DCISs, and 2 of the 16 cancers had both of these changes.

DISCUSSION

This study demonstrated that *cyclin D1* amplification and protein overexpression occur in normal tissue, breast tissue associated with increased breast cancer risk, and breast cancer. The frequencies of these changes were similar in normal tissue and epithelial hyperplasias without atypia but were higher in breast tissue showing atypical ductal hyperplasia and DCIS, reaching frequencies similar to those observed in invasive carcinoma. Experimental studies in transgenic mice showed that overexpression of cyclin D1 was associated with development of both hyperplasias and carcinomas (8). Our findings are in keeping with those results.

To date, three other studies examined cyclin D1 in benign breast disease in humans (19, 33, 34). Millikan *et al.* (33), whose study used differential PCR, did not demonstrate *cyclin D1* amplification in any of 60 subjects selected from a cohort of women with benign breast disease. There are two possible explanations for this discrepancy with our results. First, in their series, there were only 10 epithelial hyperplasias and 1 atypical ductal hyperplasia. Second, in our study, only tissue showing the specific pathologic change underwent molecular analysis, whereas in the study of Millikan *et al.* (33), localized molecular analysis was not performed. This could have decreased the sensitivity of their differential PCR, because the pathologic cells might have been diluted by noncontributory cells, such as stromal, endothelial, and inflammatory cells. In the second report, Weinstat-Saslow *et al.*

TABLE 2. *Cyclin D1* Amplification and Protein Overexpression in Normal Tissue, Benign Breast Disease, and Breast Carcinoma

Normal breast tissue			Hyperplasia without atypia			Atypical ductal hyperplasia			Ductal carcinoma *in situ*			Invasive ductal carcinoma		
Case No.	AMP[a]	IHC[b]	Case No.	AMP[a]	IHC[b]	Case No.	AMP[a]	IHC[b]	Case No.	AMP[a]	IHC[b]	Case No.	AMP[a]	IHC[b]
N1	–	–	H1	–	–	A1	+++	++	D1	–	–	C1	–	+
N2	–	–	H2	++	–	A2	–	++	D2	+++	+	C2	+	+
N3	+	+	H3	–	–	A3	–	–	D3	++	++	C3	–	–
N4	–	–	H4	+	–	A4	–	–	D4	–	–	C4	–	–
N5	–	–	H5	–	–	A5	–	–	D5	–	–	C5	–	+
N6	+	–	H6	–	–	A6	–	+	D6	–	–	C6	–	+++
N7	–	–	H7	–	–	A7	+++	–	D7	–	+++	C7	–	+++
N8	–	–	H8	–	++	A8	–	++	D8	+++	–	C8	–	–
N9	–	–	H9	–	–	A9	–	–	D9	–	–	C9	–	+++
N10	–	–	H10	+++	++	A10	+	+	D10	++	+++	C10	+	–
N11	–	–	H11	–	–	A11	–	+	D11	–	–	C11	+	–
N12	–	–	H12	–	–	A12	NP[c]	++	D12	–	++	C12	–	++
N13	–	+	H13	–	–	A13	NP[c]	++	D13	–	++	C13	–	+++
N14	NP[c]	–	H14	–	–	A14	NP[c]	–	D14	–	+	C14	–	++
N15	NP[c]	–	H15	–	–				D15	+	–	C15	–	++
			H16	–	–				D16	+	+	C16	+	+++
									D17	–	–	C17	NP[c]	–
									D18	NP[c]	+			

[a] Amplification of the *cyclin D1* gene as evaluated by different polymerase chain reaction scored as negative (–) or as +,++, or +++, representing amplification between > 1.5 and ≤ 2.5, >2.5 and ≤ 3.5, and > 3.5, respectively.

[b] Immunohistochemical studies of cyclin D1, scored as negative (–) or as + for occasional positive cells, ++ for clusters of positive cells, or +++ for diffuse immunoreactivity.

[c] No polymerase chain reaction product was detected.

TABLE 3. Association Between *Cyclin D1* Gene Amplification and Protein Overexpression

Diagnosis	% Gene amplification[a]	% Protein overexpression[b]	% Amplified cases showing immunopositivity[c]	% Immunopositive cases showing amplification[d]
Normal breast	15 (2/13)	13 (2/15)	50 (1/2)	50 (1/2)
Hyperplasia without atypia	19 (3/16)	13 (2/16)	33 (1/3)	50 (1/2)
Atypical ductal hyperplasia	27 (3/11)	57 (8/14)	67 (2/3)	33 (2/6)
Ductal carcinoma *in situ*	35 (6/17)	50 (9/18)	67 (4/6)	50 (4/8)
Invasive ductal carcinoma	25 (4/16)	64 (11/17)	50 (2/4)	18 (2/11)

[a] The numbers in parentheses indicate the number of amplified cases over the total number of cases that had a detectable polymerase chain reaction product.

[b] The numbers in parentheses indicate the number of immunopositive cases over the total number of cases analyzed, including the cases which showed no polymerase chain reaction product.

[c] The numbers in parentheses indicate the number of cases with both gene amplification and immunopositivity over the total number of cases with *cyclin D1* amplification.

[d] The numbers in parentheses indicate the number of cases with both gene amplification and immunopositivity over the total number of immunopositive cases that had a detectable polymerase chain reaction product.

(19) examined cyclin D1 mRNA expression. It is not known whether cyclin D1 mRNA overexpression is the result of gene amplification, so their results are not directly comparable to those of this study. Those investigators, however, were able to show that cyclin D1 mRNA overexpression occurred in hyperplasias with or without atypia and that there was a higher frequency of overexpression in DCISs and invasive cancers than in the hyperplasias. The third report used only immunohistochemical methods to study cyclin D1 expression. In that study, Gillett *et al.* (34) demonstrated that eight of nine atypical duct hyperplasias showed immunostaining for cyclin D1. This is a higher frequency of positivity than we observed, but this discrepancy might have arisen because different antibodies were used in the two studies, an explanation supported by the fact that they also observed a higher percentage of DCISs with cyclin D1 immunopositivity than we did.

Cyclin D1 protein overexpression in breast cancer cells, as detected by immunostaining, was reported in 28 to 81% of cases (11, 12, 16, 35–38). Our findings are within this range: 64% of our cases showed protein overexpression. There were two previous studies examining cyclin D1 immunopositivity in DCIS. One report, in keeping with our study, showed that 50% of DCISs were immunopositive (20), whereas the other report (34) showed even a higher frequency. Also, in agreement with our findings, Bartkova *et al.* (17) showed cyclin D1 immunopositivity in occasional cells in normal breast epithelium. We observed protein overexpression in the absence of gene amplification, suggesting that other mechanisms, most likely post-transcriptional in nature, play a role in cyclin D1 protein overexpression, although it is possible detection of cyclin D1 immunohistochemically might not always be indicative of protein overexpression (34). Conversely, it is not evident why protein accumula-

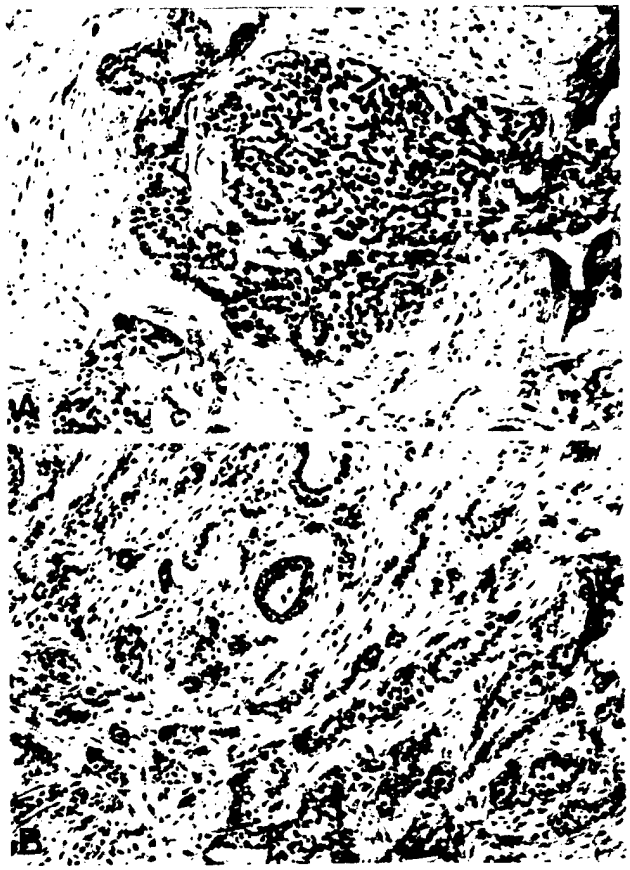

FIGURE 2. Immunohistochemical detection of cyclin D1 protein in (A) florid epithelial hyperplasia without atypia and (B) invasive ductal carcinoma. Positive nuclear staining is present (3,3'-diaminobenzidine with hematoxylin counterstain; original magnification, 400×).

tion was not observed immunohistochemically in all of the cases with gene amplification. Other authors also observed similar discordances between gene amplification and protein overexpression (16, 20). Possible explanations include changes in protein and mRNA stability, increased transcriptional rate, and method and/or antibody insensitivity.

The fact that cyclin D1 amplification and protein overexpression were detected in normal breast tissue suggests that molecular and protein changes might occur before the development of histologic changes such as hyperplasia. It is possible that the presence of amplification was an artifact of the methodology or indicative of undetected aneuploidy. We consider these explanations unlikely, however, because several reports described molecular and protein changes in apparently normal breast tissue (39–41) and because the frequency of *cyclin D1* gene amplification in invasive carcinomas in this series was similar to that detected by others with use of Southern blot analysis, which is the standard methodology (11, 38).

Cyclin D1 changes were also detected in hyperplasias with and without atypia, histologic changes associated with increased risk of developing breast cancer. The frequency of these alterations was higher in the presence of cellular atypia. The difference between the two groups was statistically significant for protein overexpression (Fisher's exact test, $P = .018$) but not for gene amplification (Fisher's exact test, $P = .662$). Our study was small, and we have no clinical follow-up, but our findings raise the question of whether positive staining for cyclin D1 will enhance our ability to predict breast cancer risk. This is of particular interest because the interobserver variability in the histologic diagnosis of atypical hyperplasia has led to questions concerning its usefulness as a marker of risk (42).

The role of cyclin D1 in the pathogenesis of breast cancer is not fully delineated despite intensive study. The low frequencies of gene amplification and protein overexpression in breast tissue showing no or minimal increased risk for breast cancer development, compared with those in breast tissue with a higher risk, suggest that changes in the *cyclin D1* gene and/or protein expression might play a role in malignant transformation. It is possible, however, that any such changes do not contribute directly to the malignant transformation of a cell but rather result in a phenotype that favors or allows the critical alterations to occur (43). For example, Zhou *et al.* (10) demonstrated that cyclin D1 overexpression in a transfected rat liver epithelial cell line resulted in increased number of cells with *CAD* gene amplification. A better assessment of the predictive significance of cyclin D1 changes in women with benign breast disease will come from prospective studies in which women with benign breast lesions are followed for the subsequent development of breast cancer. Studies of this type might also help to identify whether the presence or absence of gene amplification, the amount of gene amplification, and/or protein overexpression is the best predictor of risk of progression to breast cancer.

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Note added in proof: *Since this manuscript was accepted for publication, there has been another report describing cyclin D1 protein overexpression in normal breast tissue and benign breast disease.*

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MISCLASSIFICATION IN A MATCHED CASE-CONTROL STUDY WITH VARIABLE MATCHING RATIO

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MISCLASSIFICATION IN A MATCHED CASE-CONTROL STUDY WITH VARIABLE MATCHING RATIO

SUMMARY

We provide a simple analytic correction for risk factor misclassification in a matched case-control study with variable numbers of controls per case. The method is an extension of existing methodology, and involves estimating the corrected proportions of controls and cases in risk factor categories within each matched set. These estimates are then used to calculate the Mantel-Haenszel odds ratio estimate corrected for misclassification. A simple and conservative variance estimate is developed. An example is given from a study of risk factors for progression of benign breast disease to breast cancer, in which the risk factor is a biological marker measured with poor sensitivity.

1. INTRODUCTION

There is a considerable literature on the subject of misclassification of risk factors in epidemiological studies. The various methods are reviewed by Bashir and Duffy[1]. Methods have been developed for use in the settings of the prospective study[2], the unmatched case-control study[3-6] and the pair-matched case-control study[7-9]. In the latter case, Greenland[7,8] has developed a linear algebraic correction to the estimated numbers of case-control pairs by categories of discrete risk factors, to yield odds ratio estimates which are corrected for the effect of misclassification.

To our knowledge, a readily usable method has not been developed for the corresponding problem of a matched case-control study, a binary risk factor, and a variable number of controls per case. It is the purpose of this paper to develop such a method, prompted by a case-control study of this design in which we encountered a serious deficiency in sensitivity of detection of the risk factor of interest.

2. THE PROBLEM

Suppose we have a matched case-control study with m matched sets. Within each matched set l (l=1,2,..., m), there is one case and n_l controls. Assume we are interested in the effect of a binary risk factor. Within matched set l, let c_l be the proportion of cases with observed risk factor positive (c_l must equal zero or one), and let r_l be the proportion of controls with observed risk factor positive. If there were no misclassification, we could use conditional logistic regression to obtain the odds ratio estimate of relative risk, or equivalently calculate the Mantel-Haenszel estimate stratified by matched set:

$$OR_{MH} = \frac{(c_l(n_l - n_l r_l))/(n_l + 1)}{((1 - c_l)n_l r_l)/(n_l + 1)}$$

Now suppose the determination of the risk factor is subject to error. Clearly, if we perform the statistical analysis using the observed risk factor data, we may obtain seriously biased results[1]. If estimates of the error probabilities are available, there is scope in principle for estimating true risk factor prevalences and deriving an odds ratio estimate which is corrected for the misclassi-

fication. Greenland[7-9] develops a correction method whereby the matrix of observed cell counts is multiplied by the inverse of the product matrix of case and control misclassification probabilities to obtain estimates of the true cell counts. To expand this to the situation of multiple and variable controls per case, it is easier to lay out the calculations in terms of individual cell probabilities rather than in terms of correction by matrix multiplication. Nevertheless, the principle of back-calculation of the true risk factor prevalences within matched sets is essentially the same.

3. CORRECTING THE MANTEL-HAENSZEL ESTIMATE FOR MISCLASSIFICATION

Let RF=0 correspond to risk factor negative status and RF=1 to risk factor positive. Let OF=0 and OF=1 correspond to observed risk factor status. Using Greenland's[7] notation, we let

$$\pi_{ij} = P(OF = i|RF = j)$$

be the error probabilities for the cases and let τ_{ij} be the corresponding error probabilities for the controls. For the case in any matched set,

$$P(OF = 1|case) = P(RF = 1|case)\pi_{11} + P(RF = 0|case)\pi_{10}$$

Thus the probability that the case is truly positive for the risk factor is

$$P(RF = 1|case) = \frac{P(OF = 1|case) - \pi_{10}}{\pi_{11} - \pi_{10}}$$

Similarly for a control in any given matched set

$$P(RF = 1|control) = \frac{P(OF = 1|control) - \tau_{10}}{\tau_{11} - \tau_{10}}$$

The probabilities of being truly risk factor negative are easily calculated in the same way, as

$$P(RF = 0|case) = \frac{P(OF = 0|case) - \pi_{01}}{\pi_{00} - \pi_{01}}$$

and

$$P(RF = 0|control) = \frac{P(OF = 0|control) - \tau_{01}}{\tau_{00} - \tau_{01}}$$

From the above, we can calculate the expected number of cases positive in stratum l, say, as

$$\frac{c_l - \pi_{10}}{\pi_{11} - \pi_{10}}$$

and the expected number of controls positive as

$$\frac{n_l(r_l - \tau_{10})}{\tau_{11} - \tau_{10}}$$

We can now recalculate the corrected Mantel-Haenszel odds ratio estimate using the expected true numbers instead of the observed:

$$OR_{MHC} = \frac{\sum_l \frac{(c_l - \pi_{10})(1 - r_l - \tau_{01})n_l}{(\pi_{11} - \pi_{10})(\tau_{00} - \tau_{01})(n_l + 1)}}{\sum_l \frac{(1 - c_l - \pi_{01})(r_l - \tau_{10})n_l}{(\pi_{00} - \pi_{01})(\tau_{11} - \tau_{10})(n_l + 1)}}$$

It should be noted that in the absence of error, this simplifies to the usual Mantel-Haenszel es-

timate, and in the case of one-to-one matching to Greenland's estimate [7].

We can re-express the numerator of the above as

$$\left\{\sum_l \frac{n_l c_l (1-r_l)}{n_l+1} + \tau_{01}\pi_{10}\sum_l \frac{n_l}{n_l+1} - \pi_{10}\sum_l \frac{n_l(1-r_l)}{n_l+1} - \tau_{01}\sum_l \frac{n_l c_l}{n_l+1}\right\}$$

$$\times \frac{1}{(\pi_{11}-\pi_{10})(\tau_{00}-\tau_{01})}$$

A similar formula holds for the denominator. Decomposing the summation in this way is useful for computing purposes but has no conceptual value.

4. VARIANCE ESTIMATION

Here we develop a simple, conservative variance estimate. In the absence of misclassification, the usual variance estimate of the logarithm of the Mantel-Haenszel odds ratio is V/QR, where Q is the numerator and R the denominator of the Mantel-Haenszel odds ratio, and V is the sum of the score variances V_l for each individual stratum. The score variance for an individual stratum is in turn equal to the null variance of any arbitrary cell of that stratum[10], so

$$V_l = \frac{n_l 1 (c_l + r_l)(n_l + 1 - c_l - r_l)}{(n_l+1)^2 n_l}$$

In our point estimate after correction for measurement error, c_l is replaced by

$$\frac{c_l - \pi_{10}}{\pi_{11} - \pi_{10}}$$

and other cell values by similar formulae. The variance of c_l may therefore be replaced by

$$\frac{V(c_l)}{(\pi_{11}-\pi_{10})^2}$$

and other cell variances similarly. A conservative approximation to the overall variance would be to replace *V/QR* by *V/QRM*, where

$$M = min((\pi_{11}-\pi_{10})^2, (\tau_{00}-\tau_{01})^2, (\pi_{00}-\pi_{01})^2, (\tau_{11}-\tau_{10})^2)$$

5. EXAMPLE

We have a matched case-control study of breast cancer nested within a cohort of women with benign breast disease, with the aim of establishing risk factors for progression to cancer. We have 70 cases and a variable number of controls per case. The risk factor under consideration is the immunohistochemical marker c-erbB-2. Uncorrected risk factor status is shown tabulated by case-control status in Table 1.

The particular antibody test used for this marker in our study has poor sensitivity, given in a large validation study[11], external to our study population, as 51%. Specificity is quoted as 100%. Assuming non-differential error between cases and controls, this corresponds to $\pi_{00}=\tau_{00}=1$, $\pi_{11}=\tau_{11}=0.51$, $\pi_{01}=\tau_{01}=0.49$ and $\pi_{10}=\tau_{10}=0$. Additionally, a small repeatability study on 29 subjects in this case-control study gave the results shown in Table 2. This, together with the uncorrected control prevalence of 14% gives estimates of sensitivity and specificity of 49% and 100% respectively (details of estimation from SWD), corresponding to $\pi_{00}=\tau_{00}=1$, $\pi_{11}=\tau_{11}=0.49$, $\pi_{01}=\tau_{01}=0.51$ and $\pi_{10}=\tau_{10}=0$.

Results uncorrected for mismeasurement and corrected using the two sensitivity estimates are shown in Table 3. The corrections make little difference to the point estimate, since although they involve substantial alterations to the estimated prevalences, the alterations apply to both cases and controls. Both corrections, however, make a large difference to the interval estimate, as they entail dividing the standard error estimate by 0.51 and 0.49 respectively.

6. DISCUSSION

The method proposed here is a simple adaptation of Greenland's approach[7]. It is relatively easy to apply. While the formula for the overall estimate is awkward, its component parts are simple, and it is easy to compute. A fortran program which performs the correction is available from the authors.

When there is 1:1 matching, our point estimate reduces to that of Greenland[7], although the variance estimate does not. In the case of no mismeasurement, both our point and variance estimates reduce to the usual Mantel-Haenszel estimate and variance. Our variance estimate is relatively primitive, depending on the extent of misclassification rather than on the amount of information in the validation sample, although it is at least conservative. Indeed, it is particularly so for the example considered here, where the sensitivity is poor. The greater the maximum mismeasurement probability, the larger the corrected variance becomes. It could be argued that this is a desirable feature, giving a larger estimate of uncertainty in the case of poorer measurement.

Our example is an interesting one. From Table 3 one can see that there is a large correction to the prevalence estimates (if sensitivity is around 50% and specificity 100%, the true prevalence is likely to be around double the observed). One would normally be reluctant to make any use of a measurement which required such a large correction. It is, arguably, justifiable in this case, that of a biomarker measured by a laboratory test with well-documented false positive and negative error rates.

In principle, this method is extendable to the case of multiple levels of a risk factor and/or the effect of several covariates simultaneously. As before, the most promising approach would be to build on Greenland's method[7]. We express all possible combinations of risk factors as a single vector of dimension $d=\Pi\, d_i$, where the product is over all risk factors and d_i is the number of levels of the ith risk factor. Suppose the d x d matrix of correct and incorrect classification probabilities is M and in any given matched set the observed proportions of controls in all possible d combinations is given by the vector $\boldsymbol{p}$, of dimension d. Then we estimate the vector of

true proportions q as

$$q = M^{-1} p$$

The same formula applies to the case in each matched set, although the vector of observed proportions will have zero as every component except one, which will take the value one. This is simple in theory, but would give rise to practical problems of dealing with very large matrices if there are numerous potential confounders, and development of variance estimates would be likely to be complex.

In our example, we used both external and internal validation data to calculate the misclassificaiton probabilities. In general, it might be considered preferable to use internal validation, but with two caveats. Firstly, the correction for misclassification is applied multiplicatively, assuming independence of the validation and the main study. Secondly, it is frequently the case that internal resources enable only a small validation or repeatability study to be carried out, whereas results of large and therefore more precise validation studies may be available from the literature. In our example, there were 29 subjects in the internal repeatability study, and 187 in the external validation study[11]. Perhaps a reasonable strategy is to use information on the misclassification probabilities from both internal and external sources, as in our example.

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Table 1: Case-control status by c-erbB-2 status, uncorrected for measurement error

c-erbB2 status	No. (%) of cases	No. (%) of controls
Negative	62 (89)	235 (86)
Positive	8(11)	39(14)
Total	70	274

Table 2: First and second determinations of c-erbB-2 status cross-tabulated

First determination	Second determination	
	Negative	Positive
Negative	25	1
Positive	1	2

Table 3: Odds ratios and 95% confidence intervals unadjusted and adjusted for measurement error

Correction	Case prevalence	Control prevalence	OR	95% CI
Uncorrected (100% sensitivity, 100%specificity)	11%	14%	0.72	(0.30,1.69)
External (51% sensitivity, 100% specificity)	22%	28%	0.66	(0.12,3.50)
Internal (49% sensitivity, 100% specificity)	23%	29%	0.66	(0.11,3.71)

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p53 Protein Accumulation and Mutations in Normal and Benign Breast Tissue

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ABSTRACT

Mutations in the p53 gene are amongst the most common molecular changes detected in breast cancer and there are several reports suggesting that changes in p53 may contribute to the pathogenesis of this disease. In a previous case-control study we demonstrated that p53 protein accumulation detected by immunohistochemistry in normal or benign breast tissue was associated with a 2.5-fold increase in the risk of subsequent breast cancer. In this study we investigated whether the 29 p53 immunopositive normal or benign breast tissue samples and 15 randomly selected p53 immunonegative normal or benign breast tissue samples from the original study had p53 gene mutations. DNA was extracted from paraffin sections and underwent PCR-SSCP analysis for exons 4 to 10. PCR products that showed abnormal mobility were excised and sequenced. DNA could not be extracted from two of the immunopositive cases and they were eliminated from the study. Sixteen (59.2%) of the 27 immunopositive cases and 4 (26.7%) of the 15 immunonegative cases had p53 sequence changes. There was no obvious association between the occurrence of these alterations and any specific histopathologic features. In all, 23 gene changes were detected, and they were all base substitutions of the transition type. Ten of them were missense (amino acid change), 8 were silent (no amino acid change), 4 were intronic, and 1 was indeterminate. In summary, p53 gene alterations can occur in normal or benign breast tissue but resolution of their role in the pathogenesis of breast cancer will require long-term follow-up studies involving comparisons of breast cancer occurrence in patients with and without p53 mutations.

INTRODUCTION

Carcinogenesis is a complex multistep process which arises from the accumulation of critical genetic changes (Fearon et al., 1990; Kinzler and Vogelstein, 1996; Shackney and Shankey, 1997). The molecular changes leading to the development of breast cancer are not well characterized. However, mutations in the p53 gene are amongst the most common molecular changes detected in breast cancer (Kovach et al., 1991; Osborne et al., 1991; Runnebaum et al., 1991; Sommer et al., 1992; Coles et al., 1992; Anderson et al., 1993; Elledge and Allred, 1994; Soong et al., 1997) and several clinical and experimental studies have suggested that changes in p53 may contribute to the pathogenesis of this disease.

In experimental studies, p53 mutations occur in the preneoplastic stage of mouse mammary tumour development (Jerry et al., 1993). Recently, it has been shown that transgenic mice expressing a mutant p53 172^{R-H} minigene which had been targeted to the mammary gland developed chemically-induced breast cancer with shorter latency periods and greater tumour burden than did their non-transgenic littermates (Li et al., 1998). Gao et al. (1996) have shown that ablation of p53 function by a dominant negative p53-mutant can result in immortalization of normal human mammary epithelial cells. However, not all dominant negative mutants induce immortalization (Gao et al., 1997), suggesting that the contribution of mutant p53 to the development of cancer is complex.

In clinical studies, p53 mutations and/or p53 protein accumulation have been detected in 13 to 40% of intraductal carcinomas (Bartek et al., 1990; Poller et al., 1993; O'Malley et al., 1994; Rajan et al., 1997; Done et al., 1998; Lisboa et al., 1998;). p53 protein accumulation has also been demonstrated immunohistochemically in the benign breast tissue of patients with the Li-Fraumeni syndrome (Thor et al., 1992) and in benign tissue adjacent to breast cancer in women with a cancer

syndrome distinct from Li-Fraumeni syndrome (Barnes et al., 1992). Several reports have also shown p53 mutations and/or positive immunostaining for p53 in sporadic forms of benign breast disease (Heyderman and Dagg, 1991; Barbareschi et al., 1992; Qi et al., 1994; Millikan et al., 1995; Schmitt et al., 1995; Younes et al., 1995; Lisboa et al., 1997; Rohan et al., 1998). Collectively, these findings suggest that p53 changes can occur prior to the development of breast cancer. This is in keeping with observations by others that p53 alterations can occur in putative precursor lesions of other cancers and in normal tissues. For example, p53 mutations have been detected in Barrett's esophagus and bronchial dysplasia (Campomenosi et al., 1996; Chung et al., 1996), mutations in codons 247 and 248 have been detected in normal skin and have been shown to be associated with increased risk of developing basal cell carcinoma (Ouhtit et al., 1998), and AGG to AGT mutation in codon 249 of the p53 gene has been detected in normal liver of individuals with high exposure to aflatoxin B1, who are at increased risk of developing hepatoma (Aguilar et al., 1994).

In a previous study (Rohan et al., 1998) in which histological sections of normal or benign breast tissue were stained immunohistochemically for p53 (using the DO-7 antibody), we identified 29 subjects who showed p53 protein accumulation. One explanation for the p53 immunopositivity is that the tissue had an underlying p53 mutation. It is also possible that some of the 330 immunonegative subjects in that study had p53 mutations, since immunoreactivity can depend on the antibody used, on the type and duration of tissue fixation, or on the type of mutation, given that some mutations may not alter the protein in such a way that it can be detected immunohistochemically (Dunn et al., 1993; Jacquemier et al., 1994; Sjögren et al., 1996; Visscher et al., 1996). In relation to the latter point, one study has shown that approximately 33% of breast cancers with p53 gene mutations identified by complementary DNA sequencing did not show

positive immunostaining in tissue sections using the Cl 1801 antibody (Sjögren et al., 1996). In the study reported here, we investigated whether the 29 p53 immunopositive breast tissue samples and 15 randomly selected p53 immunonegative breast tissue samples had p53 gene mutations.

MATERIALS AND METHODS

Clinical History and Histopathology Review: Breast tissue specimens from 44 women whose biopsies showed either no histopathological change or benign breast disease were analyzed. The women selected for the study had their biopsies performed between 1980 and 1987. For each patient a representative paraffin block containing tissue from the breast biopsy was obtained. Five μm sections were cut, stained with hematoxylin and eosin, examined by light microscopy, and classified according to the criteria developed by Page and Anderson (1987).

p53 Immunostaining: As described previously (Rohan et al., 1998), 5 μm sections were cut from the paraffin blocks, mounted on aminopropyltriethoxysilane (2%, Sigma Chemical Co., St. Louis, MO) coated slides and deparaffinized, and underwent antigen retrieval (microwaved in 10 mM citrate buffer, pH 6.0, for 15 minutes at a medium-high setting). Endogenous peroxidase was inactivated using 3% hydrogen peroxide, and the sections were blocked with goat serum (20 μl/ml, Vector Laboratories, Burlingame, CA) containing 5% crystallized bovine serum albumin (BDH Laboratory Supplies, Poole, England). The sections were incubated overnight at 4°C with antibody reactive with p53 (DO-7, dilution 1:40, Novocastra Laboratories, Newcastle Upon Tyne, England). After washing, the sections were incubated with biotinylated goat anti-mouse IgG (dilution 1:200, Vector Laboratories) for 30 minutes at room temperature, followed by avidin-biotin peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories). Immunoreactivity was visualized with

3',3-diaminobenzidine tetrahydrochloride (Vector Laboratories) and the sections counterstained briefly with hematoxylin. The positive controls were sections from a paraffin-embedded breast cancer which was known to have a p53 mutation associated with p53 protein accumulation. The negative control consisted of replacing the primary antibody either with PBS or with mouse non-immune serum. The presence of nuclear staining in any number of cells seen at 100x magnification was considered a positive reaction. Cytoplasmic staining was considered nonspecific and interpreted as negative.

p53 Molecular Analysis: Five μm sections were cut from the paraffin blocks and stored for up to 3 years. Prior to microdissection the sections were dewaxed and stained briefly with hematoxylin. The epithelium in the region of the tissue which had shown p53 immunoreactivity was microdissected out and placed in a microfuge tube. The tissue sections which showed no p53 protein accumulation immunohistochemically underwent random microdissection of epithelium. The tissue was digested with proteinase K (0.5 mg/ml in 50 mM Tris HCl, pH 8.5, 10 mM EDTA, 0.5% Tween 20) for at least 48 hrs at 55°C (Zhu et al., 1998). The proteinase K was inactivated by heating at 95°C for 15 min.

An aliquot of the digest was amplified using PCR, [α-^{33}P]-dATP and exon-specific primers (see Table 1). An aliquot of the reaction product was separated on an 8% non-denaturing polyacrylamide gel and the gel was processed for autoradiography (Orita et al., 1989; Murakami et al., 1991). Potential mutations were detected by shifts in band mobility. If no band shifts were detected in these samples, the tissue was considered to have no mutation. For samples showing band shifts, the PCR-SSCP analysis was repeated. If the two PCR-SSCP analyses generated different band shifts, another section was cut, microdissected and processed for PCR-SSCP analysis as

described above. Negative controls including cells which contained no mutation and a blank water control were included in each analysis. In addition positive controls for exons 5 to 9 (exon 5:SKBr 3; exon 6:T47D; exon 7:colo 320 DM; exon 8: MDAMB468; exon 9: SW480) were also included where appropriate. The cell lines used as positive controls had been embedded in agar, fixed in 10% formalin, and paraffin-embedded to simulate the processing conditions of the breast tissue.

The abnormally shifted band was excised from SSCP gels and the DNA was eluted into water. The DNA was reamplified by PCR using the same primers and the product was run on a 2% agarose gel. The band was extracted using QIAquick gel extraction kit (Qiagen Inc, Mississauga, ON). The purified DNA was sequenced using ThermoSequenase radiolabelled terminator cycle sequencing kit (Amersham Life Sciences, Cleveland, OH) and the sense primer according to the manufacturer's directions, followed by gel electrophoresis and autoradiography. To confirm the mutation, the DNA product was resequenced using the antisense primer. Negative controls were included in each analysis. Cell lines with known mutations in exons 5 to 9 were also included where appropriate. Gene alterations were compared to those listed for breast cancer in a p53 database (http://www.iarc.fr/p53).

RESULTS

For two of the immunopositive cases, we were unable to extract DNA and these cases were eliminated from the study. Of the 42 cases from which we could extract DNA, 22 showed fibrocystic change, 8 showed adenosis with or without fibrocystic change or fibrosis, 8 had hyperplasia (mild, moderate, or florid), 2 had fibroadenomas, and 2 showed no histopathological change. Table 2 summarizes the findings of the immunohistochemical staining and molecular gene analysis for p53

according to the histological features. Of the 27 cases with p53 immunopositivity, 16 (59.2%) had p53 sequence changes. Ten of the 11 cases with p53 mutations showed p53 immunopositivity. Of the 15 women whose biopsies were immunonegative, 4 (26.7%) had p53 sequence changes. There was no obvious association between the occurrence of gene alterations and any specific histopathologic features. A representative photomicrograph of a seetion stained for p53 is shown in Figure 1 and a representative SSCP gel and its corresponding sequencing gel are shown in Figure 2.

Exons 4 to 10 were analyzed for mutations. For all cases except one, the SSCP changes were reproducible. In the one case (case 24) where the SSCP change was not reproducible, the repeat analysis had been done on DNA extracted from a different section and only wild type DNA sequences were seen on the second analysis. In all, 23 sequence alterations were detected in 20 individuals. The changes found were all base substitutions of the transition type (Tables 3 and 4). Two mutations occurred at CpG dinucleotide sequences (cases 2 and 24). Two mutations occurred at known hot spots on the p53 gene, one at codon 175 and the other at codon 245. Of the 23 gene changes, 10 were missense mutations (amino acid change), 8 were silent (no amino acid change), and 4 were intronic. The remaining gene change occurred in case 5, for which an abnormal pattern for exon 9 was detected in the SSCP gel, while all the other exons showed wild type patterns. Although the sequencing pattern could not be interpreted because of the presence of numerous extra bands, this sample was still considered to have a mutation. Missense mutations were distributed amongst exons 4, 5, 7 and 9, silent changes were detected in exons 4, 6 and 7, and the intronic changes were in introns 6, 7, and 9. Three individuals had two sequence changes each; in two of them both changes were silent and in the third, one of the two resulted in an amino acid change. Of

the 4 intronic alterations, 2 were in the same location (nucleotide residue 14766) in intron 9 and showed the same change (t→c). The others were at nucleotides 13466 in intron 6 and 14114 in intron 7. None of the intronic mutations occurred at a splice site or created a new splice site. In addition to the results shown in Tables 3 and 4, the known p53 polymorphism in codon 72 (CGC→CCC) was detected in 2 cases.

Of the 18 changes in exons, all of them occurred in codons identified in the p53 breast cancer database as having mutations. Four of them showed the same base and amino acid change as has been identified in breast cancer. A similar comparison could not be done for the intronic mutations because the nucleotide residues of the intronic mutations are not provided in the database. Similar to those reported in the p53 database, most base substitutions in this study were G to A and C to T transitions (IARC p53 mutations database http://www.iarc.fr/p53).

For all individuals with a p53 gene alteration, the adjacent stromal tissue underwent microdissection and extraction of the DNA. The exon which had been identified as abnormal in the epithelial cells was analyzed by PCR-SSCP. In 18 of 20 cases, wild type p53 banding patterns were observed (Figure 3). In the other two (cases 29 and 34) the same gene alteration was present in the stromal cells as in the epithelial cells.

DISCUSSION

Disruption of p53 function appears to have a pivotal role in carcinogenesis (Wang and Harris, 1997; Hussain and Harris, 1998). p53 is involved in regulating cell proliferation, inducing apoptosis, and promoting chromosomal stability (Ko and Prives, 1995; Levine, 1997). In relation to the cell cycle, p53 is involved in maintaining cells in G0 (De Sal et al., 1995), and in regulating

progression of cells from the G1 to the S phase (Kuerbitz et al., 1992; Levine, 1997) and possibly from the G2 to the M phase (Agarwal et al., 1995; Cross et al., 1995; Ceraline et al., 1998). p53 has also been implicated in DNA repair as it can bind to DNA helicases and may modulate nucleotide excision pathways (reviewed in Wang and Harris, 1998; Huang, 1998). It is also involved in regulating DNA replication and chromosomal segregation (Dutta et al., 1993; Gualberto, 1998). p53 protein is a transcription factor, and to date, at least 6 genes have been identified which contain p53-dependent, *cis*-acting, DNA-responsive elements and which have a role in effecting the various functions of p53 (Ko and Prives, 1995; Levine, 1997). Changes in p53 might contribute to carcinogenesis by conferring a proliferative advantage to cells with or without abnormal DNA, and/or by facilitating the accumulation of additional genetic changes, for example by allowing aneuploidy and genetic instability to occur (Shackney and Shankey, 1997). To date, it is not known at which stage in the carcinogenic process p53 abnormalities develop.

Our results demonstrate that p53 gene alterations can be detected in breast tissue which is either normal or shows changes of benign breast disease. p53 changes were found more commonly in tissue which showed p53 protein accumulation (positive immunostaining) than in tissue that did not. All of the changes detected were of the transition type. This is in keeping with experimental data showing that DNA proofreading corrects transversions more efficiently than transitions (Schaaper, 1993).

There have been three other reports of p53 gene analysis in normal or benign breast tissue. Millikan et al. (1995) detected p53 point mutations in 5 of 60 paraffin-embedded breast samples. Two of the mutations occurred within the 14 cases which were immunopositive for p53 and the other three occurred in the 46 immunonegative cases. All of the mutations were transition types and three

resulted in an amino acid change. In contrast to our study, their analysis involved only exons 4 to 8 which may provide a partial explanation for the lower frequency of mutations in their study. Lisboa et al. (1997) detected a p53 mutation in one of 13 cases of normal or benign breast tissue examined. However, they examined only exons 5 to 8 inclusive and did not perform microdissection and so may have missed mutation(s) present in only a small number of cells. Done et al. (1998) identified 7 cases of breast cancer from which they were able to microdissect 41 foci of surrounding normal epithelium or epithelium showing changes of benign breast disease. The p53 gene analysis was performed on DNA extracted from paraffin-embedded tissue. They did not detect any mutations, but their study was based on a small number of cases, and only exons 4-8 were studied.

In the present study, sequence changes occurred in 59.2% of p53 immunopositive samples. Although this value may appear low, it is in keeping with the findings of several studies of breast cancer which have examined the correlation between immunostaining and the presence of mutations detected by sequencing (Dunn et al., 1993; Jacquemier et al., 1994; Sjögren et al., 1996; Visscher et al., 1996). In those studies, 16 (Dunn et al., 1993) to 70% (Visscher et al., 1996) of immunopositive breast cancers showed mutations. Our value may in part reflect the fact that we considered the presence of any p53 immunopositivity to represent a positive case whereas it has been suggested by others that only cases showing immunopositivity in greater than 5% of cells should be considered to have p53 protein accumulation (Clausen, 1998). Alternatively, the p53 protein accumulation may be due to mechanisms other than p53 mutation. Four (26.7%) of our p53 immunonegative cases showed gene alterations. It is not surprising that p53 changes were detected in the absence of positive immunostaining as it is well accepted that not all p53 mutations will result in immunohistochemically-detectable p53 protein (Schaaper, 1993; Hurlimann et al., 1994; Baas

et al., 1994; Visscher et al., 1996).

Several features of our study suggest that the mutations that were detected were real and were not artefacts of the methodology used to detect them. It has been shown that PCR-induced sequence changes can be minimized if the proteinase digestion time of the tissue is sufficiently prolonged (at least 48 hrs), the products generated by PCR are relatively small (Shiao et al., 1997), and enough DNA template is used (Krawczak et al., 1989). In our study the tissue was digested for at least 48 hours and the products were all less than 300 bp in size. Although we were unable to quantify the amount of DNA in each analysis, a fixed cycle number was used in the PCR for each exon of all samples and it was not increased if the product was undetectable. Secondly, repeat PCR-SSCP analysis showed that the band shifts were reproducible. The one case where it was not reproducible, the analysis had been done on DNA extracted from a different section and it is likely that the area with the p53 change was no longer present. The abnormal band was sequenced in both directions to ensure that the sequence change was not a PCR-induced artefact and in each case the same mutation(s) was detected. Thirdly, DNA from stromal tissue showed wild type p53 sequences in 18 of 20 cases. The other 2 cases (29 and 34) had the same mutation in both epithelial and stromal DNA. For these cases, the changes might represent inadvertent microdissection of some epithelial cells with the stromal tissue or a true germline mutation or a polymorphism. We consider it more likely that the change detected in these two cases is a polymorphism because it has been detected in approximately 4% of tumours in a breast cancer tumour bank (H. Ozçelik, unpublished data). Fourthly, we were able to detect a known polymorphism in two other cases. Finally, other studies such as that of Nadji et al. (1996) have shown that DNA extracted from paraffin-embedded tissue will show p53 gene changes identical to those detected in frozen tissue, suggesting that paraffin-

embedding does not induce gene mutations, and that tissue processed in this way is suitable for DNA analysis. Eight of the 23 gene changes that were detected were silent. Strauss (1997) predicted that approximately 25% of mutations in a dataset will be silent if mutagenesis is random and if the silent mutation does not provide a selective advantage. Although the significance of silent mutations is not known, it is possible that they could have effects on DNA.

In conclusion, the results of this study suggest that p53 mutations can be detected in normal epithelium and benign breast tissue. This observation is in keeping with the findings of other studies demonstrating genetic changes such as loss of heterozygosity (LOH) and microsatellite instability in normal and benign breast tissue (Deng et al., 1996; Lakhani et al., 1996; O'Connell et al., 1998; Larson et al., 1998; Lininger et al., 1998). However, the significance of p53 mutations in these tissue types is currently unknown. For example, a recent study showed that genetic changes such as LOH and microsatellite instability may not correlate with the development of breast cancer (Kasami et al., 1997). For skin, however, it has been suggested that p53 mutations may provide information about subsequent risk of developing nonmelanoma skin cancer (Ouhtit et al., 1998; Nakazawa et al., 1999). Resolution of the role of p53 gene alterations in the pathogenesis of breast cancer may require long-term follow-up studies involving comparisons of breast cancer occurrence in patients with and without p53 mutations and assessment of the functional significance of the mutations.

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FIGURE LEGENDS

Figure 1

Photomicrograph of normal breast ducts showing p53 immunopositivity (immunoperoxidase with hematoxylin counterstain, magnification x 160).

Figure 2

(A) Representative SSCP gel of exon 9 PCR product from four cases. Case 34 shows a band shift as indicated by the arrow. The negative control which had wild type p53 (C-) and positive (C+) control (cell line SW480) are included. **(B)** The corresponding sequencing gel of case 34 shows a base substitution (t→c) as indicated by the arrow. The sequencing pattern for the negative control (C-) in the same region is also shown.

Figure 3

Representative SSCP gels of exon 4 (case 44), intron 7 (case 17) and exon 9 (case 16) showing DNA which had been extracted from epithelial cells (E), from corresponding stromal cells (S) and the appropriate negative control (WT). The DNA from the epithelial cells show a different band pattern than the corresponding stromal and negative control DNA.

Table 1 p53 Primer Sequences and PCR Conditions

Primers	Sequences	Product size (bp)	PCR Conditions
exon 4[a]	5'-ATCTACAGTCCCCCTTGCCG-3' 5'-GCAACTGACCGTGCAAGTCA-3'	296bp	95°C, 50sec; 55°C, 50 sec; 72°C, 60sec, 35 cycles
exon 5[b]	5'-GCTGCCGTGTTCCAGTTGCT-3' 5'-CCAGCCCTGTCGTCTCTCCA-3'	294bp	95°C, 50sec; 58°C, 50sec; 72°C, 60sec, 30 cycles
exon 6[b]	5'-GGCCTCTGATTCCTCACTGA-3' 5'-GCCACTGACAACCACCCTTA-3'	199bp	95°C, 50sec; 55°C, 50sec; 72°C, 60sec, 30 cycles
exon 7[b]	5'-TGCCACAGGTCTCCCCAAGG-3' 5'-AGTGTGCAGGGTGGCAAGTG-3'	196bp	95°C, 50sec; 56°C, 50sec; 72°C, 60sec, 30 cycles
exon 8[b]	5'-CCTTACTGCCTCTTGCTTCT-3' 5'-ATAACTGCACCCTTGGTCTC-3'	225bp	95°C, 50sec; 55°C, 50sec; 72°C, 60sec, 30 cycles
exon 9[c]	5'-GCCTCAGATTCACTTTTATCACC-3', 5'-CTTTCCACTTGATAAGAGGTCCC-3'	152bp	95°C, 50sec; 56°C, 50sec; 72°C, 60sec, 30 cycles
exon 10[a]	5'-TGTTGCTGCAGATCCGTGGG-3' 5'-GAGGTCACTCACCTGGAGTG-3'	130bp	95°C, 50sec; 55°C, 50sec; 72°C, 60sec, 33 cycles

[a]reference (Mashiyama et al. 1991)
[b]reference (Millikan et al. 1995)
[c]reference (Mazars et al. 1992)

Table 2 Summary of Analyses of p53 Immunohistochemically Detected Protein Accumulation and Gene Changes According to Histological Features

Histology	# of samples	# of samples I+[a] M+[b]	# of samples I+ M-	# of samples I- M+	# of samples I- M-
Normal	2	2	0	0	0
FCC[c]	22	9	5	2	6
Adenosis[d]	8	1	4	1	2
Hyperplasia[e]	8	3	2	1	2
Fibroadenoma	2	1	0	0	1
TOTAL	**42**	**16**	**11**	**4**	**11**

[a] I+ = immunopositive, I- = immunonegative
[b] M+ = gene change present, M- = gene change absent
[c] FCC = fibrocystic change
[d] adenosis = adenosis ± FCC ± fibrosis
[e] hyperplasia = either mild, moderate or florid

Table 3 p53 Mutations in Breast Tissue

Case No.	Location	Site	Sequence Change	Amino Acid
24	Exon 4	codon 72	CGC→CGT	Arg→Arg
	Exon 4	codon 110	CGT→TGT	Arg→Cys
46	Exon 4	codon 76	GCA→ACA	Ala→Thr
2	Exon 5	codon 175	CGC→CAC	Arg→His
4	Exon 5	codon 135	TGC→TAC	Cys→Tyr
28	Exon 5	codon 133	ATG→GTG	Met→Val
36	Exon 5	codon 178	CAC→CGC	His→Arg
3	Exon 7	codon 245	GGC→GAC	Gly→Asp
9	Exon 7	codon 227	TCT→TTT	Ser→Phe
40	Exon 7	codon 244	GGC→GAC	Gly→Asp
5	Exon 9	NI*		
16	Exon 9	codon 325	GGA→GAA	Gly→Glu

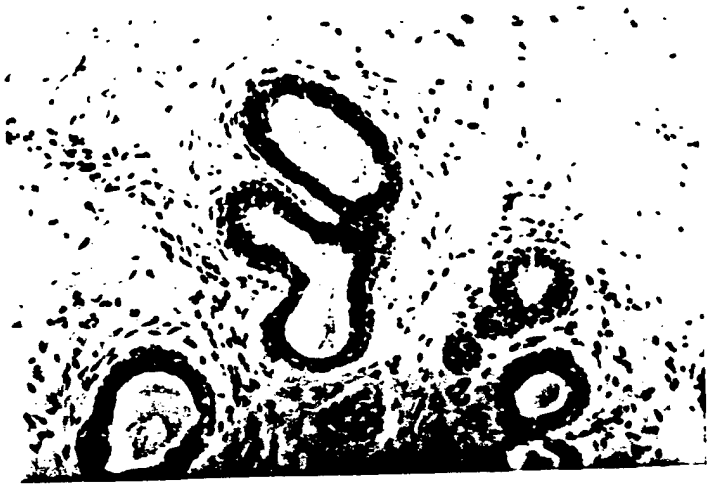
*NI= not interpretable

Table 4 p53 Changes That Do Not Cause Amino Acid Changes

Case No.	Location	Site	Sequence Change	Amino Acid
Silent change				
32	Exon 4	codon 74	GCC→GCT	Ala→Ala
	Exon 4	codon 111	CTG→CTA	Leu→Leu
44	Exon 4	codon 111	CTG→CTA	Leu→Leu
18	Exon 6	codon 217	GTG→GTA	Val→Val
27	Exon 7	codon 231	ACC→ACT	Thr→Thr
		codon 239	AAC→AAT	Asn→Asn
48	Exon 7	codon 226	GGC→GGT	Gly→Gly
Intronic change				
26	Intron 6	nr 13466	g→a	
17	Intron 7	nr 14114	g→a	
29	Intron 9	nr 14766	t→c	
34	Intron 9	nr 14766	t→c	

nr = nucleotide residue

Figure 1



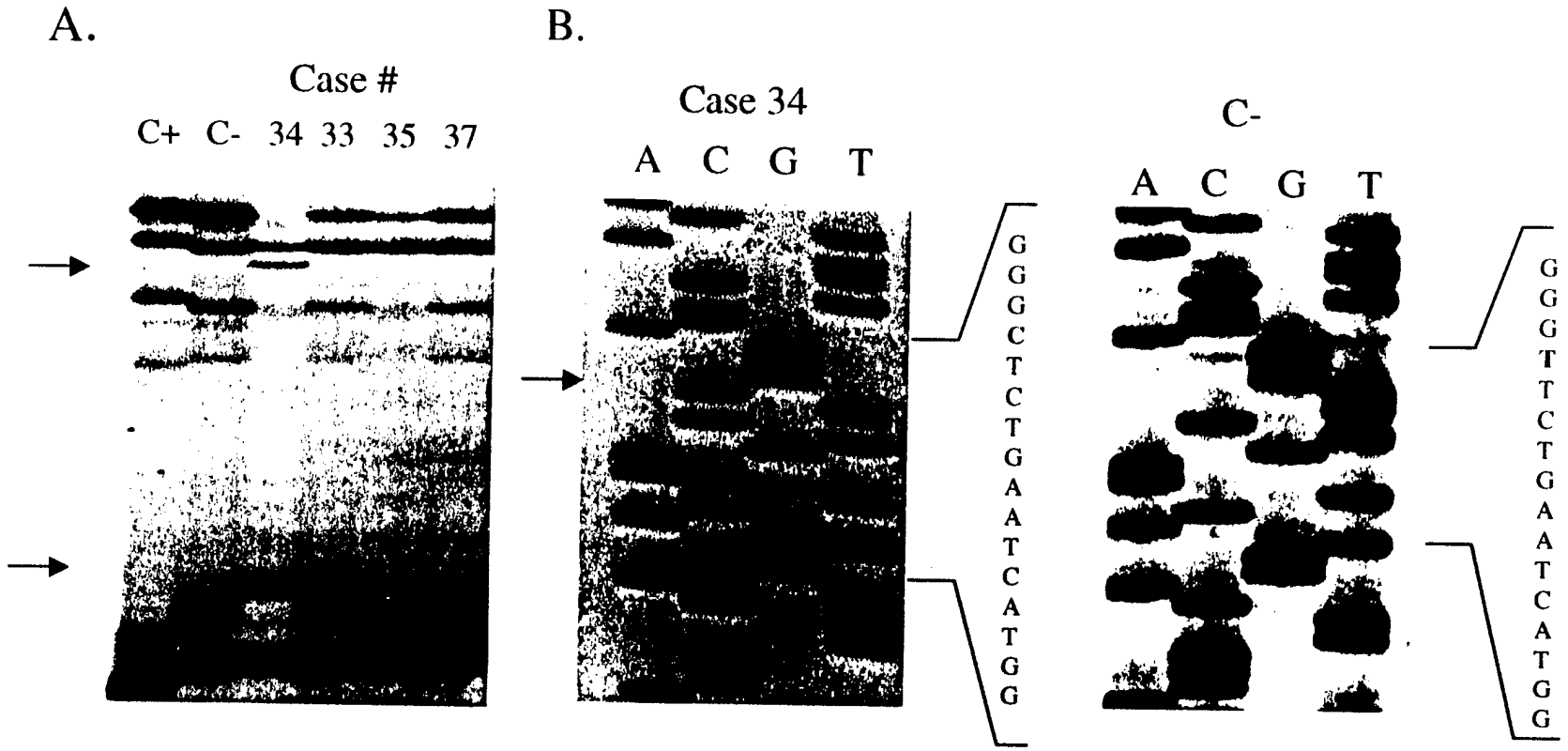

A.
Case #
C+ C- 34 33 35 37
B.
Case 34
A C G T
G G G C T C T G A A T C A T G G
C-
A C G T
G G G T T C T G A A T C A T G G

Figure 2

Case 44

Exon 4

WT S E

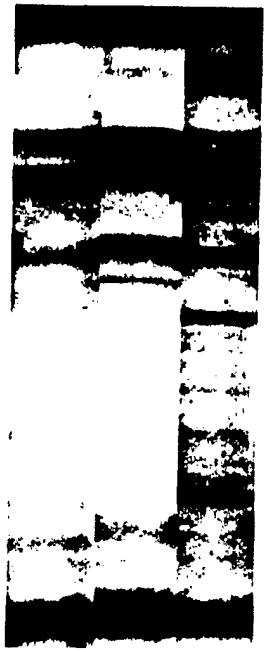
Case 17

Intron 7

WT S E

Case 16

Exon 9



WT S E

Figure 3

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Cyclin D1 in benign breast disease and risk of breast cancer. Rohan, T., Zhu, X-L., and Kandel, R. *Dept. of Public Health Sciences, University of Toronto, Toronto, Ontario M5S 1A8; Dept. of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario M5G 1X5, Canada.*

Cyclin D1 plays a role in regulating cell cycle progression and appears to have a role in carcinogenesis. We studied the association between cyclin D1 overexpression in benign breast disease (BBD) and risk of progression to breast cancer. The investigation was conducted as a case-control study nested within the cohort of 4,888 women in the National Breast Screening Study (NBSS) who were diagnosed with biopsy-confirmed BBD during active follow-up. Case subjects were women with BBD who subsequently developed breast cancer. Control subjects were matched to each case on NBSS study arm, screening center, year of birth, and age at BBD diagnosis. We analyzed paraffin-embedded benign breast tissue sections from 70 case subjects and 287 control subjects for cyclin D1 protein overexpression immunohistochemically. Cyclin D1 protein overexpression was not associated with increased risk of progression to breast cancer (adjusted odds ratio = 1.13; 95% confidence interval = 0.58-2.24). However, this does not preclude a role for cyclin D1 gene amplification, analysis of which is currently ongoing.

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Comparison of c-erbB-2 (HER-2) Status in Cytologic Specimens and Corresponding Tissue Sections of Breast Carcinoma

Charalambos Solomides, M.D.,
Robert Zimmerman, M.D., and **Marluce Bibbo,** M.D.

INTRODUCTION/PURPOSE: To determine any significant difference in the c-erbB-2 immunoreactivity between cytologic smears and the corresponding tissue sections of breast carcinoma.
MATERIALS AND METHODS: Alcohol-fixed smears were made from 36 fresh mastectomy/lumpectomy surgical pathology specimens from breast carcinomas. Immunohistochemistry was performed using the c-erbB-2 primary antibody against the extracellular domain of the c-erbB-2 gene product. Staining was simultaneously performed on formalin-fixed, paraffin-embedded tissue sections of the same specimens. Slides were graded independently by three authors, and agreement was reached by consensus, with majority agreement.
RESULTS: A total of 36 cases were reviewed. Positive immunoreactivity was determined for c-erbB-2 in 27 (75%) of alcohol-fixed specimens (n = 14[3+] and n = 13[2+]) and 19 (53%) of formalin-fixed, paraffin-embedded tissue specimens (n = 11[+3] and n = 8[+2]).
CONCLUSION: C-erbB-2 expressions in fresh cytologic material is significantly higher ($P < .05$) than in the corresponding formalin-fixed, paraffin-embedded tissue. This is a sensitive and simple processing method that can be routinely applied to fresh surgical pathology specimens or fine needle aspiration biopsies for the detection of c-erbB-2 (HER-2). The high rate of immunoreactivity of c-erbB-2 in cytologic specimens is comparable to recent published data regarding HER-2/new gene amplification in cytologic specimens by fluorescence *in situ* hybridization (FISH). A direct comparison between these two methods could be of great interest.

From Jefferson Medical College and Presbyterian Medical Center, Philadelphia, Pennsylvania, U.S.A.

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Detection of p53 Mutations in ThinPrep® Processed Fine Needle Aspirates of Breast Carcinoma

Aaron F. Pollett, M.D., **Yvan C. Bédard,** M.D., Ph.D., and **Rita A. Kandel,** M.D.

INTRODUCTION/PURPOSE: Molecular analyses of tumor cells obtained from fine needle aspirates (FNA) may provide prognostic information and allow early assessment of responsiveness to chemotherapy. In this study we examined ThinPrep® (Cytyc Corporation) processed FNAs of breast cancers for the presence of p53 mutations and compared the results to conventionally processed surgical specimens.
MATERIALS AND METHODS: DNA was extracted using TRIzol® (Gibco BRL) from 47 breast FNAs reported as suspicious or positive for malignancy. As well, DNA was extracted from 20 corresponding formalin-fixed, paraffin-embedded tumors after microdissection using proteinase K digestion. PCR followed by single strand conformation polymorphism (SSCP) and sequencing was used to examine for mutations in exons 4 to 9 of the p53 gene.
RESULTS: p53 mutations were detected in 10 tumors. They were predominantly point mutations and involved all exons except 8. In 8 of the 10 patients both surgical and cytology analysis was available, and 6 of the 8 cases showed identical mutations. In the two remaining cases mutations were present only in the paraffin-embedded tissue.
CONCLUSION: ThinPrep processed breast FNAs provide, with less processing time, DNA suitable for p53 molecular analysis. When p53 mutations were detected in the FNA, these were identical to those detected in paraffin-embedded tissue.

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